

What to do about new herbicides

THE British government has made a muddle for itself over the herbicide 2,4,5-T, rightly held by farmers to be one of the best ways of ridding fields of nettles, thistles and other weeds. Since the middle of the Vietnam War, it has been known that commercial preparations of the material are potentially dangerous because of the small quantities of dioxin which they contain. The Seveso accident dramatized the potential danger of the contaminant. Since then, the British government (like many others) has taken steps to limit the concentrations of dioxin permitted in manufactured lots of the herbicide, but by doing so has appeared merely to excite the morbid interest of trades unions and their members. In recent weeks, unions with as little naturally in common as the National Union of Agricultural and Allied Workers (NAAAW) and the Association of Scientific and Managerial Staffs (ASTMS) have taken up cudgels against this apparently beneficent chemical (*Nature*, 10 July), asking that its use should be stopped. The government's capacity to make a rational response, or even to decide what to do, has been entirely undermined by the belated and shamefaced confession by the Minister of Agriculture on 20 June that his department has misled not merely itself but other bodies to which it has proffered reassuring advice by its mistaken estimate of the amounts of 2,4,5-T used in Britain.

The toxicology of the dioxin and of 2,4,5-T are by no means precisely catalogued, but some of the bare bones of the story are simple. Dioxin, an exceedingly stable chemical, causes chloracne in largeish doses but is also teratogenic and is known also to be carcinogenic. So much is clear from laboratory studies but also, unhappily, from accidents in which human beings have been put at risk. Laboratory studies have also shown that 2,4,5-T itself may be a teratogen. Whether it is also carcinogenic at doses which are not also acutely toxic remains an open question. But it is beyond doubt that dioxin is a much more potent teratogen than the pure herbicide. Such laboratory evidence as exists is unfortunately incomplete. The Advisory Committee on Pesticides (PAC) of the Ministry of Agriculture, Fisheries and Food quoted in March 1979 data which suggest that mice and hamsters suffer teratological consequences only at doses which are also toxic. Doubt has however been cast on this conclusion, so that there are some who say that the effective teratological doses of the two materials are related by a factor of 10^{-4} or thereabouts. If this should be the case and because the permitted concentration of dioxin in otherwise pure chemical 2,4,5-T is one part in 100 million, the foetus of a pregnant woman 'eating commercial 2,4,5-T by the spoonful is more likely to be damaged by the principal ingredient than by the dioxin contaminant. The recognition of this possibility has come as a shock to many observers of the scene, and to the NAAAW in particular. It is of course ironical that the government, by taking steps to limit the damage that might be done by the contaminant in a commercial herbicide, should have served only to focus attention on the potential dangers of the herbicide itself.

None of this suggests that 2,4,5-T as used in Britain (or elsewhere that intensive agriculture is practised) should be considered an immediate danger. The total amount of the active ingredient used in Britain appears (whereby hangs a tale) to be of the order of 100 tonnes a year, something of the order of 100 grams a year for each of, say, a million agricultural workers. The current recommendation of the Food and Agriculture Organization and the World Health Organization is that 24 milligrams per day entails no risk of teratogenic damage. Even if

British agricultural workers perversely chose not to spray all the herbicide on nettles but, rather, ate it or fed it to their pregnant wives, a substantial fraction of the total quantity now used would have to be diverted in this way before widespread damage was caused. Because most of the herbicide used does actually end on nettles and not in people's cups of tea, because 2,4,5-T is distinguished from dioxin by its relative instability and because the indirect consequences of the use of the material are likely as a consequence to be small, it is hard to think that harvested food can be seriously contaminated.

So why is there so much fuss? The most serious issue is that questions have been raised about the basis for the recommendations of PAC about the safe levels of 2,4,5-T. The question is by no means an easy one to settle. Such data as are to be found in the literature are not part of systematic and controlled studies, while many of them derive from the early 1970s when the need to distinguish between the effects of dioxin and 2,4,5-T was not fully appreciated (and the technical capacity to distinguish between them analytically was only meagre). Yet there is some reason to suspect that the recommendations of PAC are not as solid as they might have been. This, no doubt, is one of the issues that the several government committees concerned, PAC itself but also the committees of the Health and Safety Executive (HSE), will be trying to resolve before the summer is out. Nobody should be surprised if it turns out that the available data are insufficient for an informed conclusion to be possible.

Whatever the outcome, it is inevitable that the mere shadow of doubt cast on the recommendations of PAC will call into question the constitution of that body, which is one of those vaguely defined entities of which British bureaucracy is proud to boast. The committee is for practical purposes responsible for licensing the uses made of pesticides. Its positive recommendations have the force of legal licences. Negative recommendations are the equivalent of bans. To outsiders, the great anomaly in the constitution of PAC is that manufacturers are not required by law to submit new pesticides, or new formulations of old pesticides, to the committee. Compliance has always been, and remains, voluntary. Even so, to the surprise of many people, the system has worked well and imaginatively — the regulation of the organochlorine pesticides has, for example, been sensible. Equally surprising, there is no evidence that manufacturers deliberately keep new products from the eyes of the committee — the fear that there would in that case be a statutory scheme is enough to keep them all on the straight and narrow path. Such loopholes in the British arrangements for the regulations as there are centre on the import of materials manufactured overseas but not recommended by PAC, but again there is no evidence that this is substantially abused. The danger now, for the Advisory Committee on Pesticides, is that if it should turn out that it has erred on the side of what may seem to be complacency in such an important (or at least controversial) matter as 2,4,5-T, the pressures for converting from a voluntary to a statutory scheme will be almost irresistible. That would mean, for all concerned, more bumbledom.

The second issue that will keep the civil servants busy through the summer is that the Ministry of Agriculture has grossly underestimated the quantity of 2,4,5-T being used in Britain. For the past two years, the ministry has been saying that the quantity was something like three tonnes a year, largely on the basis of a survey by major users, the Forestry Commission in particular. Quite inexplicably the ministry, proud as it is of its connections



with farmers through the Agricultural Advisory Service, failed to appreciate that the chief use of 2,4,5-T is for spraying crops and grassland to get rid of weeds. But even the revised estimate of 50 tonnes a year has had to be doubled to more than 100 tonnes in the light of figures supplied by Customs and Excise of the quantities of the herbicide imported. The broader issue here is that the ministry has for several years resisted complaints that its surveys of the use of pesticides on British farms are adequate.

What, while these questions are being resolved, are the unions (and their members) to do and think? By all accounts, PAC will make a detailed investigation of the specific allegations of damage done to people that have been mentioned by the agricultural workers, but that cannot be a quick job. What the union must do is what it should have been doing all along — to make sure that workers spraying pesticides wear adequate protective clothing.

Mr Clive Jenkins of ASTMS is frying other fish. To his usual preoccupation with the recruitment of members, he appears to have added a wish to back the Health and Safety Executive rather than the Ministry of Agriculture as the arbiter of what pesticides are safe. His inclinations are understandable, for union representation on HSE is laid down by law. In reality, however, both executive and ministry have a finger in the protection of agricultural workers, while HSE is solely responsible for the occupational risks of chemical plants where 2,4,5-T may be manufactured. Even Mr Jenkins is on solid ground when he argues that the executive has dealt ineptly with recent problems. Whatever the strength of these complaints, however, it would be a great misfortune if the use of a herbicide whose value is unquestioned were to be needlessly and prematurely banned simply because the government has made a hash of this affair.

Nato, France and the neutron bomb

WHILE many parts of France enjoy the spectacle of protests against the building of civil nuclear power stations, there has been hardly a complaint at the French government's announcement that one of its recent nuclear tests was that of a neutron bomb. Just possibly the explanation is that French taxpayers accept the theory of strategic deterrence hook, line and sinker, and are persuaded that French neutron bombs are intended never to be used but that the civil nuclear power stations being built in France are meant to be used, and quickly. It is, however, more probable that the silence which has greeted the announcement of the neutron bomb betokens a general agreement by the French population with the national strategy which has been working out since the mid-1950s. Whatever the explanation of the domestic reaction, opinions appear to have been much changed by the new development. The *Süddeutsche Zeitung* was saying last week, for example, that the neutron bomb means that France is no longer a token nuclear power. What does it all mean?

For the best part of a quarter of a century, France has been the odd man out in European defence. The roots of the present policy appear to go back to Gaullist days, and to the theory advocated by General Jean Gallois that each nation should possess a nuclear striking force somehow commensurate with its own importance to a potential adversary. In reality, they probably have more ancient origins. It is too easily forgotten that the French have long memories, as long perhaps as those of the Irish. The French experience of two world wars is a sufficient justification of the conviction that nation states should in the last resort be masters of the means of their own defence. The painful withdrawal of France from NATO was as much a reflection of this belief as of overt distrust of the assurances in the NATO treaty or of disagreement with NATO strategy. Only this can properly explain how, even with the passage of two decades, France remains on the sidelines, now consulting closely with NATO and behaving as if a full-blown member, but stolidly opposed to rejoining the club.

From this point of view, the development of the neutron bomb is logical enough. So long as France holds to the view that the time may come when the defence of France by Frenchmen (without help from elsewhere) may be necessary, it plainly makes sense that France should be equipped with those nuclear weapons which, at least in theory, could be used tactically in European circumstances without inviting massive retaliation from the other side. Whether, of course, the financial cost of this new investment is worthwhile, only the government of France can judge. Apparently, however, the taxpayers most immediately concerned have no objection. Two years or so from now, which is the soonest the new weapons could be deployed, France may thus be the only nuclear power to risk deploying these new weapons in a theatre where their use makes sense.

The consequences for states on both sides of the German border are not easy to foresee. The most comforting calculation is that France will equip itself with only a modest force of neutron

bombs, one so modest that it could have no decisive influence in a major European conflict. Certainly there has been no protest yet from the East at what the French are planning that compares with the trouble evoked when the United States announced its intention to deploy neutron weapons in Europe more than a year ago. Perhaps the President of France was able to stave off trouble when he met Mr Brezhnev in Warsaw last month, or perhaps the protest is yet to come. Whatever the future, however, it seems inevitable that the latest developments in France will change the regard of other European states for the non-NATO member among them. But this time, unlike the early 1960s, France will tend to be regarded not as the black sheep of Europe but as a symbol of the implicit belief now strengthening in Europe that European responsibility for its own defence is not merely prudent but necessary. The Gallois doctrine, after a long hiatus, is catching on.

So is there a chance that in the years ahead Western Europe as a whole will accept that it must, in the last resort, be responsible for its own defence? And what will be the consequences? The tendencies in this direction which have become apparent in the past few years differ markedly from those which carried France out of NATO twenty years ago. There are few in Western Europe who now doubt the commitment of the United States to European defence. Their anxiety, rather, is that this commitment has been one of the few reliable components of a foreign policy which has as often mystified as reassured. Escapades such as the abortive attempt to rescue the American hostages in Iran are worrying for Europeans not so much because of the direct risk of a conflagration, limited though it might well have been, but because they cast doubt on the machinery for consultation within NATO and on the process of policy formation in Washington. These doubts will not go away when the American election has been decided. There are also, however, other forces tending to strengthen European hankerings after a European defence policy. It becomes increasingly anomalous that the European Community has only the most slender means for formulating policy towards the rest of the world — the brief declarations tagged on to communiques such as that issued from Venice a few weeks ago are no substitute for a foreign policy. Moreover, it becomes ever more apparent that Western Europe is evolving its own sense of priorities as to how relationships with the East should be conducted. Chancellor Schmidt's visit to Moscow, sceptically regarded at the outset by the United States, has nevertheless opened the possibility of constructive discussions about medium-range missiles in Europe that could, if by some remote chance they succeeded, be a great boon to the industrialized West and East alike. On the face of things, the French neutron bomb (not yet a weapon) is a gesture of national independence, but it will curiously also help to strengthen Western Europe's growing tendency towards coherence and independence.

Congress supports technology centres

Washington

Plans are now being advanced to implement one of the key proposals made by the Carter Administration last year to help stimulate technological innovation — the setting up of a number of “generic technology” centres. The proposal has so far escaped budget cuts in Congress.

The purpose of each “generic technology” centre would be to carry out research and development in a particular area of technology underlying a range of industrial needs, rather than being related to any specific product. Typical examples given include welding, tribology, the study of new composite materials and the general area of automated manufacturing.

Officials at the Department of Commerce are now putting together detailed proposals from industry to establish three or four such centres. The hope is that, if Congress can be persuaded to allocate the \$5 million requested for the programme, initial work on the centres would be under way by the beginning of the next fiscal year on 1 October.

The idea behind the centres is that the government should provide seed money to stimulate research of a type which no individual company may be prepared to support, but which is likely to benefit groups of companies or whole industries. Each centre would be jointly funded by industry, initially with the government picking up most of the bill, but with government support dropping to 20 per cent or less after five years.

Each centre, according to details published by the department last month, will have responsibilities in three main areas: first, to maintain a substantial in-house research and development effort; second, to provide a range of technical services, including consultants, information, training facilities and technology evaluation; and, finally strategic planning of technology development.

The idea has already found many sympathetic ears in the US Congress. The Senate, for example, recently passed an innovation bill, originally proposed by Senator Adlai Stevenson, Jr, chairman of the Science and Space Subcommittee, aimed specifically at encouraging research links between industry and universities by setting up a number of “centres for industrial technology”. The Senate bill has already been accepted in principle by the Science and Technology Committee of the House of Representatives.

The private sector is also keen. Technology centres were one of the chief recommendations of the Research and Development Advisory Panel to last year's domestic policy review of innovation. The committee recommended that direct federal support of new technology generic to process or product innovation in wide spectra of industries “should be

strengthened and recognized as federal policy”. Its chairman, Dr Jack Goldman of the Xerox Corporation, remains enthusiastic about the Stevenson bill.

Some controversy remains, however. For example, should the universities be research contractors for groups of companies setting up a particular generic technology centre, as the Department of Commerce seems to be recommending? Or should universities be given a more prominent role from the start as the point of contact between government and industry?

The second strategy has been advocated by the National Science Foundation, which has set up a number of university-based co-operative research centres under a programme initiated in 1973. The most suc-

cessful of these has been the Polymer Research Center at the Massachusetts Institute of Technology, now self-financing from industry sources with an annual budget of \$600,000.

NSF's own plans for expanded efforts in this direction had to be trimmed by \$2 million when the Administration revised its budget request to Congress in April. But NSF officials are convinced that their approach, using universities as “surrogates” for federal contact with industry, has several advantages over what one refers to as the Commerce Department's “pie in the sky” efforts to forge ambitious links between government and industry virtually from scratch.

David Dickson

Carnesale for NRC chairman

Washington

Dr Albert Carnesale, professor of public policy at Harvard University and deputy director of the Center for Science and International Affairs there, was nominated last week by President Carter for the newly-upgraded chairmanship of the Nuclear Regulatory Commission (NRC). Dr Carnesale is a nuclear engineer by training, with experience of both arms limitation and non-proliferation negotiations. This, together with his reputation as a “moderate” on nuclear issues, has led both anti-nuclear and pro-nuclear groups to greet his appointment with a cautious welcome.

Carnesale's involvement in nuclear energy debates, for example as a participant in the Ford-Mitre study of 1976 and as head of the technical coordinating committee for the US delegation to the International Nuclear Fuel Cycle Evaluation (INFCE), nevertheless suggests that his views are closely aligned with the Administration's position on sensitive issues such as nuclear reprocessing and non-proliferation policy.

Dr Carnesale received a master's degree in engineering at the Drexel Institute in Philadelphia, and subsequently a PhD at North Carolina State University. After five years working as a nuclear engineer with the defence contractors Marietta Martin, he joined the Arms Control and Disarmament Agency, and was a senior adviser to the US delegation which negotiated the first Strategic Arms Limitation Treaty (Salt I) in 1972.

He returned to North Carolina State University as head of the division of environmental studies in 1972, subsequently moving to Harvard in 1974, where he now teaches a class at the Kennedy School on “Technology, War and Peace”, while remaining a government adviser on various defence and nuclear issues.

Dr Carnesale refuses to be drawn on specific ideas about the current problems

of the NRC, which has been under considerable strain following its handling of the Three Mile Island accident and its aftermath last year. But he said last week that he considers the safety of existing reactors to be “the first item of business for anyone”.

On the reprocessing of spent fuel, whose deferral by President Carter is now under renewed attack in Congress, Dr Carnesale points out his agreement with the conclusion of the Ford-Mitre study (subsequently accepted by INFCE) that at present there is no economic case for recycling fuel from thermal reactors.

He considers the INFCE exercise to have been a “success” in generating an international dialogue on nuclear fuel cycle issues, even though it failed to endorse many aspects of US policy. “It has to be judged in a far wider perspective than the reports themselves”, he says. But he refuses to be drawn on whether the United States, as some of the more ardent congressional supporters of its non-proliferation policy had argued, should have taken a bolder stance in registering areas in which it disagreed with the final consensus.

Waiting for approval . . .



This is one area where Dr Carnesale's views are likely to be closely examined by Congress when it considers his nomination. The NRC's refusal to grant an export licence for nuclear fuel to India has already prompted moves to circumscribe its responsibilities for reviewing exports as detailed by the Non-Proliferation Act signed in 1978. Legislators are soon to consider whether to veto President Carter's decision to overrule the NRC and grant the licences. But they will want to know how sympathetic Dr Carnesale is to the proposals of Mr Gerard Smith, head of the INFCE delegation, that some of the more stringent provisions of the NNPA be amended.

Dr Carnesale's appointment is said to have been actively canvassed within the White House by the Office of Science and Technology Policy, keen to defuse what has recently been called "the most politicized agency in town" — as well as to see strong leadership given to a body which, prior to the Three Mile Island accident, relied more heavily on collegial decision-making by its five commissioners.

David Dickson

Policy analysis

UK adventure?

Can pure reason throw light on the relationship between technical innovation and social change, assisting the first and softening the consequences of the second? This is the inspiration of the brave gamble launched last week in London by the Leverhulme Trust, the Science Research Council (SSRC). Between them, the three organizations plan to spend £2.55 million setting up an organization known as the Technical Change Centre. Sir Michael Swann, the retiring chairman of the BBC, will run the governing body of the new centre, but a director has yet to be found.

The centre is intended as an instrument of policy analysis; it will seek, in other words, to influence events. Its sphere of operations was last week defined more confidently by example than by precept — it may look into public perceptions of the

hazards of nuclear power, opportunities in but also the social consequences of microelectronics and the question whether academic training at the postgraduate level is good or bad for British industry. The sponsors' interest in the gamble is best explained by Sir Michael Swann's plaintive question why Britain, the leader in technological innovation for two centuries, should have failed so dismally since the Second World War.

The centre has venerable antecedents. Four years ago, the SSRC flirted with the notion of setting up in Britain an equivalent of the Washington-based Brookings Institution, but failed to find charitable partners for that venture. The new venture is in part a survivor of that plan, but with the difference that the SRC is an equal partner with the SSRC, that the Leverhulme Trust will contribute 60 per cent of the cost of the first five years and that the terms of reference, still broad, have been made more narrow.

Last week, the sponsors of the centre were frank in their estimate of their success. Much will depend on the director they can recruit; they also confess that they may be beaten by the intractability of the problems they tackle. They plan to make a cool appraisal of their venture (which may get under way by the beginning of 1981) in about four years. If things are going well, they would hope to give it at least a second lease of life.

Precisely how the centre's work will evolve plainly awaits the appointment of a director. For Sir Michael Swann emphasized last week that its policy will evolve independently of the interests of the sponsors. He explained, however, that the SSRC has a special interest in policy analysis of this kind more or less as an end in itself, while the SRC has a more specific interest in receiving advice on how its own budget should be disposed.

The core of the governing body has already been recruited, and includes the chairmen of the two sponsoring research councils (Sir Geoffrey Allen and Mr Michael Posner), Dr Duncan Davies (Chief Scientist at the Department of Industry), Sir Alastair Pilkington (soon to retire as chairman of Pilkington Brothers, the glassmakers) and Mr Philip Hughes (chairman of Logica Limited, a software house). It is intended that the board, when complete, should include representatives of trades unions.

Sir Michael Swann says that the absence of the Agricultural and Medical Research Councils from the list of sponsors has no significance, but plainly the new centre will be looking to them as well as to other organizations for funds with which to carry out sponsored research; the funds so far available amount to just over £500,000 a year, but the centre would hope to recruit a further £200,000 a year of soft money. While most of its work will be carried out in-house, it will also support work at other centres in the United Kingdom, thus no

doubt helping to ensure that it is not regarded too much as a threat by existing academic centres.

Teaching companies

Victims of success

The British Science Research Council and the Department of Industry are in difficulties about the financing of their joint teaching company scheme. Paradoxically, their problems have arisen because the scheme is proving more successful than originally foreseen.

When the teaching company scheme was set up five years ago, the aim was to secure the participation of about 20 companies by 1980. Interest has been such, however, that 33 companies are now involved and many more are hoping to join. The SRC and DoI agreed to fund the scheme on a fifty-fifty basis but the rapid expansion has meant that this year the DoI may not be able to pay its share. The total cost of the scheme has risen from £0.5 million in 1979 to an estimated £1.5 million this year, of which the DoI can contribute only £600,000.

The SRC is confident that it will be able to take up the slack in 1980; the real problems will arise in future years, especially if expansionary pressures persist. The SRC's contribution could probably increase to about £1 million a year, but other sources of finance would then have to be found. A panel of industrialists, civil servants and academics is to be set up at the end of this month, with a remit to make recommendations by the end of the year.

The objective of the teaching company scheme is to introduce industry to the skills universities have to offer and at the same time to provide recent graduates with industrial experience. Typically, a company agrees to take on three recent graduates, called associates, in each of the first two years of membership.

The SRC and DoI between them pay the associates' basic salaries, incidental expenses, cost of any special equipment they need and the cost of any replacement staff needed in universities. The company's major expense is usually that of implementing recommendations for improvements.

The pressures to expand the scheme come not only from companies wishing to join but also from those already in the scheme who say they have found it so useful that they are reluctant to leave. So far, only two companies have actually left the scheme. Because of the pressure on funds, the SRC is now beginning to talk to several companies about ways of terminating formal membership.

Both industry and universities seem to be enthusiastic about the scheme. Industry effectively gets a free consultancy service and is able to tap otherwise inaccessible resources. Universities have the opportunity to put some of their academic work into practice.

Judy Redfearn

Swann is the boss



Technological forecasting

Rush programme

The European Commission's FAST project, a five-year programme in science and technology forecasting set up in 1979, is up to its eyes in applications for funds. Invitations to tender for part of the £1.35 million budget were sent out early this year; and by the deadline of 10 April some 224 applications had been received from 176 institutions. Research projects relating to Europe 10, 20 and 30 years ahead must be completed by mid-1982, although the 30-40 successful applicants will be officially notified only in September.

FAST's director, Ricardo Petrella, says this compression of the timetable is inevitable, given that the programme was funded for only five years by a decision of the Council of Research Ministers on 25 July 1978. The FAST team of six scientists and four assistants spent most of 1979 defining the nature of the programme. The research phase of the programme now beginning must end during 1982 and early 1983 with the specification of research and development priorities for Europe the ultimate object of the exercise.

For the participating universities and research institutes, there will be little time to come to grips with what are complex issues — "microelectronics innovations in the context of the international division of labour", for example, and "social acceptability of biotechnology-based activities". This may account for what one observer outside the commission has called the unimaginative nature of most of the applications.

The applications are also biased to short-term issues. Petrella's team distinguished three broad sectors of problems — work and employment, the role of information technologies and the role of biotechnology — which Petrella identifies as of greatest importance over periods of 10, 20 and 30 years respectively. Some 100 applications were received for research in the first sector, 70 in the second but only 35 in the third. The final selection of projects will, however, be more uniformly divided.

The short perspective of the FAST applications might have mattered less if a parallel proposal within the European Science Foundation had come to fruition. The plan had been that the ESF, an independent body uniting national research councils throughout Europe, should have mounted an "additional activity" on "the socio-economic consequences of technical development", paid for by those among its members wishing to participate. This proposal, which would have been concerned with more basic and less policy-orientated objectives than is FAST, was thrown out by the ESF's social science committee last year.

One of the objectives of FAST, defined by the 1978 European Council decision, is

"to establish an *ad hoc* system of collaboration between specialized research groups within the community and so create a series of community forecasting networks". All very well — but what can be done on a European scale that is not already being done nationally? ("Send me the answer on a postcard", one UK government science adviser asked one of the FAST team.) It is a question of scale and regionality, according to Mark Cantley, head of the FAST biotechnology programme. Investigation of the manpower and training implications of biotechnology-based industries, for example, requires consideration of international transfers and coordination. The distribution of benefits and risks associated with microelectronics and its applications is similarly a regional matter.

FAST itself is also under test, however. The earlier, small-scale "Europe plus 30" study recommended the establishment of a Community Forecasting Institute. The council rejected that in 1978 and instead set up FAST for a provisional five years. Petrella and his team naturally hope that the experiment will be continued.

Robert Walgate

Soviet energy

No shortage?

The Soviet Union and its socialist allies have no energy crisis ahead, according to Petr Neporozhnyi, the Soviet Minister of Energy and Electrification. This confidence, he said, was based on estimates of the Soviet Union's fuel reserves — more than 50 per cent of the world reserves of coal and more than 33 per cent of the world reserves of oil and gas. Furthermore, the Soviet Union's hydroelectric capacity is potentially larger than that of the United States, Canada and Japan taken together.

The minister was speaking on the English-language service of Moscow Radio beamed to North America. Inevitably, he compared Soviet energy policy with that of the United States. The latter, he said, has in its oil shales "more than several Saudi Arabias" — but the "American monopolies" find it more profitable to import oil and raise retail prices of oil products than to develop new deposits. The minister glossed over the fact that one of the main barriers to the use of shale is environmental pollution. Estonia, which burns much shale in thermal power stations, is a major source of pollution in the Baltic area.

Neporozhnyi's confidence is to a large extent supported by the latest figures from PetroStudies (Malmo). This independent team monitors Soviet oil and energy exports on the basis of trade figures with individual countries. According to the report for 1979, published last week, the Soviet Union is now the second largest oil exporter in the world (next to Saudi Arabia), exporting 164 million tonnes in

1979, and for the past six years has been the world's leading oil producer, with an annual target for 1980 of 606 million tonnes. The same confidence was shown by Dr Mikhail Tolkachev of the Soviet Academy of the National Economy, reporting to a meeting of the International Institute of Applied Systems Analysis earlier this month. This year, said Tolkachev, Soviet natural gas production is expected to reach 4.2 million m³ per day, an increase of more than half a million m³ since last year.

Soviet claims to energy sufficiency are not new. Neporozhnyi's talk, however, is interesting in that it virtually ignored nuclear power, except for a passing mention of plans for integrating the Soviet grid with those of the various Comecon countries and the construction of the new ETL-750 transmission line to connect Rzeszow in Poland with the Khmel'nitskaya atomic power station in the Ukrainian SSR. This is the more surprising since Neporozhnyi put considerable stress on the Soviet planners' "concern for the future" — as exemplified in the long-range energy plan launched 20 years ago with the construction of the Bratsk power station in Siberia. The same concern, said the minister, has led to long-term plans for the production of synthetic liquid fuel, the use of solar and geothermal energy and even "the heat of ordinary sea water".

To most Soviet energy planners, however, nuclear power is usually a key topic in any speech on energy prospects. In April 1979, Academician Anatolii Aleksandrov, president of the Soviet Academy of Sciences, and himself formerly a leading figure in the nuclear energy programme, stressed that it was atomic energy and coal which would help the Soviet Union to avoid what he called the "imminent energy crisis". The Soviet nuclear programme does not, however, appear to be keeping up with the demand.

Last month, at an All-Union conference on nuclear power held, appropriately, at Energodar, Sapozhnikov, one of Neporozhnyi's deputy ministers, stated that the construction of nuclear power stations must be doubled and new commissioned capacity raised to 7,000-10,000 MW per year. The target is an overall nuclear generating capacity of 100,000 MW within the next 10-12 years.

According to Fedor Ovchinnikov, another deputy minister of energy, the short-term programme is for water-moderated water-cooled reactors of up to 1,500 MW capacity. Officially at least, there are no anxieties on environmental grounds. Yurii Markov, a spokesman for the Ministry of Energy and Electrification, wrote recently in *Sovetskaya Rossiya* that Soviet nuclear power stations are so safe that their surroundings "are used as recreation areas, where families come and picnic, gather mushrooms and berries and go fishing in the cooling ponds".

Vera Rich

Technological change

Worry about jobs

Washington

The new report on science and technology policy (see *Nature*, 10 July, page 96) published by the Organization for Economic Cooperation and Development (OECD) is being cold-shouldered in Washington largely because of its warning that undirected technological change may be contributing to the rising level of unemployment. The report, prepared by a group of outside experts, sets out to identify the main elements of the social and economic environment for science and technology which are likely to affect OECD countries during the 1980s.

The report emphasizes the need to revitalize technological innovation in order to help remedy many of the afflictions of the 1970s, especially sluggish economic growth and escalating inflation. To this uncontroversial conclusion, however, it adds that technological innovation must be guided so as to ensure that its potential benefits are not overshadowed by the destruction of jobs.

The extent to which technical change, in both manufacturing and service industries, may be contributing to growing unemployment, expected to reach 8.5 per cent next year, is receiving more and more attention, particularly at state and local levels. Pennsylvania's legislature, for example, is considering a bill intended to reduce unemployment by banning automatic petrol stations and toll collection booths, among other measures.

In Washington, by contrast, the government resists the notion that it must act to soften the impact of technical change on employment. Last year's domestic policy review of industrial innovation, for example, concluded merely that "the key to successful adjustment is warning time"; and the national Labor Technology Forecasting System subsequently promised by President Carter has yet to materialize. The dominant argument is that once rapid economic growth has been re-established, jobs lost through technical change in one sector of industry will be replaced elsewhere. In any case, there is growing antagonism towards government intervention in the regulation of innovation, so that the lack of enthusiasm for the OECD report is no surprise.

The report started as an internal study within OECD to examine the relationship of technical change to the problems of inflation, unemployment and long-term economic growth. In part, it was intended as a counter-analysis to the official OECD report "Towards full employment and price stability", produced by a committee chaired by Professor Paul McCracken and published in 1977.

The new report challenges the McCracken thesis at two related levels. First, it argues that the economic problems

faced by OECD countries in the early and mid-1970s were not, as McCracken suggested, merely a manifestation of cyclical economic changes that could be resolved through conventional policy instruments. Rather, the new report argues, they reflected deeper structural changes requiring a more radical approach.

More specifically, the report challenges the McCracken committee for underestimating the impact of technical change on social and economic policy, and for characterizing the declining rate of innovation as a problem that will resolve itself "naturally" with a revival in demand. Instead, the report argues that adequate policies for science and technology must be central to any attempt to tackle the joint problems of high inflation and unemployment. These problems, it argues, tend to depress the rate of innovation; but a slackening of innovation makes their solution more difficult.

Government action is therefore necessary, the report argues, to ensure both a faster and a "more balanced direction" for technical change. But in the United States, where most government economists accept the McCracken thesis, this has met with a certain scepticism. A particular source of irritation has been the report's warning that a general substitution of capital-intensive for labour-intensive techniques in manufacturing industry may be exacerbating unemployment.

Professor Richard Nelson of Yale University, a member of the OECD expert group, admits that the committee which prepared the report was "schizophrenic" on the employment issue. Thus it argues that "a significant acceleration of technical advance and productivity growth would make the unemployment problems easier to deal with" but also warns that more capital-intensive technology may further worsen the condition of the labour market.

The report adds that structural unemployment is now an "important feature of the economic landscape". The hypothesis is an increasingly familiar one in Europe, particularly in Britain where it has been argued forcefully by, among others, Professor Christopher Freeman of the Science Policy Research Unit at the University of Sussex, a member of the group which prepared the OECD report. Economists here prefer to point to similar concerns in the late 1950s and early 1960s that the automation of production might create serious unemployment; in the event, excess labour was absorbed into the rapidly-growing service sector.

Academic economists tend to be more agnostic than their government colleagues. Dr Robert Lund of the Massachusetts Institute of Technology, who has examined studies carried out at Sussex on the impact of microelectronics, says that "we did not find the kinds of employment impacts that seemed to be predicted". But he adds that "I do not know if we have enough data to

say that we have the right answers".

Government policy-makers are less equivocal. The OECD report came in for substantial criticism when a draft was circulated last year. Strenuous, and ultimately successful, efforts were subsequently made within OECD to ensure that the report was published as the opinion of a group of experts rather than with the organization's official backing.

The net result, however, is that the report's potential impact on policy has been blunted, which Professor Nelson for one regrets. "On the employment question, it may not be quite right, but it is more correct than some of the discussions now taking place."

David Dickson

Thermonuclear fusion

Soviets bid half

Brussels

The cost of the International Test Fusion Reactor (INTOR), estimated at \$1,000 million, would be more than halved if the machine were built in the Soviet Union. This is the opinion of Dr L G Golubchikov, head of the fusion division of the Soviet State Committee on the Utilization of Atomic Energy who has been seeking to stimulate a greater commitment to the project from the West during his participation in the eighth international conference on plasma physics which ended last week.

The project, which so far consists largely of a design study, is being planned by the International Atomic Energy Agency (IAEA). American reaction at the conference, however, was cool. It might cost half as much, but it would take twice as long, was one opinion.

INTOR, whose study by the IAEA was proposed by the USSR in May 1978, is nevertheless agreed to be a constructive exercise in one respect: by concentrating attention on the severe engineering difficulties of the tokamak fusion reactor, it is helping to define the experimental questions which must be tackled in the next generation of large tokamaks (JET in Europe, T-15 in the USSR, TFTR in the United States and JT-60 in Japan). However, it is also generally agreed (except by the USSR) that the INTOR design is unlikely to see the light of day as a piece of hardware, at least in the medium term.

What appears most likely is that the recent proposal (*Nature*, 10 July) for a Fusion Engineering Device (FED) in the United States at a hardware cost of \$1,000 million will capture (and double) the US fusion budget. FED, largely the work of the Princeton Plasma Physics Laboratory, is less ambitious than the Oak Ridge National Laboratory's proposal, the Engineering Test Facility (ETF), which in turn was less ambitious than INTOR. Moreover, it is felt in the US Department of Energy that collaboration with the USSR on a project of the scale of INTOR would be fraught with bureaucratic difficulties.

Japan would also consider collaborating with the United States on FED although it is a little early for formal approaches to have been made. Professor Husini, a Japanese representative on the IAEA's International Fusion Research Council, said last week that Japan would be "very interested" in such a joint venture, on the basis of sharing costs.

This leaves the European Community in the cold beyond JET, unless it also joins in FED, which is by no means out of the question. The European Commission has been considering the next European Torus (NET) in a small committee headed by the Italian physicist Professor R Toschi of Frascati; but, said one Italian, "the FED recommendation means NET will have to be completely rethought. Perhaps we can build a device a step beyond FED and five years later."

The USSR would clearly be disappointed if INTOR goes little further than the project definition, which is to be presented to the IAEA next year. "The West has the bigger energy problem" said Golubchikov. The Soviet authorities are thus less bullish about fusion. It would thus assist the Soviet fusion community greatly if the next steps could be international. And Golubchikov believes the technical problems that will face FED or INTOR will be so great that Soviet participation will be necessary if there is to be rapid progress. After all, the USSR invented the tokamak — which appears to be the only viable ignition device at present — and built the only working superconducting tokamak (T7).

Robert Walgate

Laboratory merger Harwell again

Several large research facilities in Britain will be completed late, and Britain's capacity to contribute to new international projects will be impaired, as a result of pressures on the budget of the Science Research Council. This is the view of Sir Geoffrey Allen, chairman of the SRC, speaking at an Open Day at the newly-merged Rutherford and Appleton Laboratories last week. Although the science budget has been let off relatively lightly in recent public expenditure cuts, he said, a 10–15 per cent increase of the budget would be needed to complete existing projects within the original timetables and to start new ventures.

One victim of these enforced delays is the Spallation Neutron Source, at present under construction at Chilton, across the fence from Harwell. It will now be completed in 1984, two years later than originally planned, largely because it has been necessary to spread out the funding of the £12 million project. Allen is also concerned that Britain may not be able to contribute fully to the building of a new European synchrotron radiation source (*Nature*, 20 May) or to the European Space Agency's plan to build an astrometry

satellite, Hipparcos.

The proposed European synchrotron radiation project, devised by the European Science Foundation, would require a new international agreement among the partners; Allen plainly fears that the SRC budget may be too tight to allow Britain to contribute. Hipparcos presents a different problem. It will be paid for out of ESA's mandatory science budget to which all member states, including Britain, contribute annually on the basis of GNP. Allen's



concern here is that British laboratories should recapture some of the cost by making viable bids for the ESA contracts.

The merging Appleton and Rutherford Laboratories are hoping to overcome this manpower problem by training and re-deploying people as their projects come to an end, or as timetables are extended. The laboratories claim some success in these directions, but acknowledge that there must be limits to the extent to which staffs with different, highly specialised backgrounds, for example in radio and space physics (Appleton) and high-energy physics (Rutherford), can be fully redeployed.

A major reason for the merger is to save costs, including some staff. The SRC hopes to shed about 40 administrative jobs by not replacing those who choose not to move to the new site. Most of the scientific staff are expected to move, however, and those who do not will be replaced. So far about a third of the 300 Appleton staff have moved. The rest will do so before the end of 1981.

The plan to close the site of the Appleton laboratory at Ditton Park cannot be implemented precisely as originally hoped, however. Some of its instruments will not be compatible with the Chilton site; some millimetre wave and radio equipment, for example, will be sited 60 miles away to avoid interference from the spallation neutron source and 70 MeV linac.

Judy Redfearn

Siberian science

Marchuk on top

Last month's meeting of the Supreme Soviet confirmed the appointment of Academician Guri I Marchuk as head of the State Committee for Science and Technology. The confirmation was a formality; Marchuk's appointment took effect last January, when the then head, Academician Vladimir A Kirillin, resigned (coincidentally on the day that Academician Andrei Sakharov was exiled to Gor'kii).

Marchuk came to the State Committee from the Siberian branch of the Academy of Sciences, of which he had been chairman since 1975. That branch, based on the science centre of Akademgorodok outside Novosibirsk, was originally set up, in 1957, as part of Khrushchev's overall plan to decentralize the economy. Its activities have always, therefore, been closely related, at least formally, to the needs of Siberia.

The Siberian branch would therefore share the blame if science failed to play a full part in the opening up of the Siberian heartland. In the autumn of 1976, criticism of the Siberian branch did in fact break out. The State Monitoring Committee at the outset criticized the Academy of Science's Far Eastern Science Centre for failing to make a proper practical contribution to the national economy. The campaign turned to more and more prestigious institutions, finally attacking the Siberian branch itself. At that point, Marchuk fought back, and with an impressive battery of arguments demonstrated that, so far from slacking, the branch was alive and flourishing and more than pulling its weight.

In his new job as head of the State Committee, Marchuk is responsible for the whole of Soviet science planning, the coordination of research and development and the subsequent implementation of research. Marchuk's Siberian experience will be invaluable, for Siberia remains the major area of Soviet capital investment.

Last April, a special conference of the Central Committee of the CPSU advocated increased investment in the fossil-fuel-bearing regions of West Siberia, apparently because a lack of amenities is making it hard to attract scientists and engineers to the area. The new Baikal-Amur Mainline railway was planned from the beginning as a joint exercise in engineering, ecology, demography and economics.

Plans to divert the major Siberian rivers south were, however, delayed during the last five-year plan, although, in response to complaints at the possible global environmental consequences, the Soviet government announced that no such changes would occur. It has however mounted a major environmental study of the region, with the result that Siberian

environmentalists have had unparalleled opportunity for field work in the past decade. This has involved the photography of the area from manned and unmanned spacecraft, ground-level geophysical surveys (which on one occasion discovered glaciers far to the south of where they were expected) and the drawing up of ecological guidelines for the engineers. There must be no tree-felling on slopes of more than 15

degrees and no insecticides — construction gangs plagued by the ubiquitous Siberian mosquitoes must use protective netting or swatters.

In spite of all this care, a recent report from the Chita Hydrometeorological Observatory suggests that the development of the BAM zone is running into environmental problems. In the north Transbaikalian area, it appears, there is very little wind; the

air masses remain static for a considerable time and industrial emissions become concentrated. Long before the BAM railway itself is officially opened, the pollution in the Chara valley is already, say the Chita scientists, some 50-100 per cent greater than that in the European part of the USSR — the centre, at present, of Soviet industry.

Vera Rich

CORRESPONDENCE

Biko's doctors

SIR, — The Faculty of Medicine of the University of the Witwatersrand has strongly attacked the decision of the South African Medical and Dental Council that there was no evidence of improper or disgraceful conduct on the part of the doctors who treated Mr Steve Biko before his death in detention. The Faculty Board has resolved unanimously to dissociate itself publicly from the council's decision, taken at a closed meeting on 17 June 1980.

At a meeting held on Friday, 27 June, the Board of the Witwatersrand University Medical Faculty unanimously adopted a resolution on the "Biko doctors". The resolution reads: "The Faculty of Medicine of the University of the Witwatersrand, Johannesburg, has considered the decision of the South African Medical and Dental Council, taken at a closed meeting on 17 June 1980, that there was no evidence of improper or disgraceful conduct on the part of the doctors who treated Mr Steve Biko before his death, and that there was thus no need for a disciplinary hearing."

"Bearing in mind the revelations under cross-examination at the inquest, and the fact that the magistrate presiding at Mr Biko's inquest had considered that the matter of the Biko doctors should be referred to the South African Medical and Dental Council, the Faculty expresses its deep concern and disquiet at the finding that there was no evidence of improper or disgraceful conduct and that the matter should be dropped. The faculty feels that there was *prima facie* evidence of improper or disgraceful conduct, which should have been subjected to the careful scrutiny of a Medical Council Disciplinary Committee hearing."

"The Faculty is of the opinion, furthermore, that this decision of the Medical Council may seriously affect the good standing of the South African medical profession both at home and abroad and, therefore, publicly dissociates itself from this decision."

Further, the Faculty endorses and supports the Guidelines for Medical Doctors concerning Detainees and Prisoners, adopted by the 29th World Medical Assembly of the World Medical Association in Tokyo in October, 1975, and known as the "Declaration of Tokyo". This states, *inter alia*, that a doctor must have complete clinical independence in deciding upon the care of a person for whom he or she is medically responsible (Article 5), and that a doctor shall in all circumstances be bound to alleviate the distress of his fellow men, and no motive — whether personal, collective or political — shall prevail against this higher purpose (Article 8).

"The Executive of the Board of the Faculty of Medicine is requested to ensure that the views of the Board are communicated widely to the public and to the medical community, both in South Africa and abroad."

In accepting this resolution, the Faculty stressed that "We are not saying that the doctors are guilty. We are saying that there is sufficient evidence for a Disciplinary

Committee enquiry to be instituted and that a decision should be taken about the behaviour of the doctors on the basis of a full, fair, impartial and thorough Disciplinary Committee hearing. The South African Medical and Dental Council is supposed to be the watch-dog of the ethics of our profession and they have been zealous — and some would suggest over-zealous — in the severity of the punishment that they have meted out, not only for major infringements but for what many would regard as minor infringements of the ethics of medical practice. Yet, in the present case, it is difficult to accept that the Medical and Dental Council has applied its collective mind to the problem of the Biko doctors in a purely objective and dispassionate way."

In moving the resolution, Professor Clive Rosendorff, Head of the Department of Physiology at the Medical School, made this statement:

"This is all a source of great embarrassment and distress to many doctors in South Africa who are proud of the high ethical standing of their profession, both within this country and abroad. I believe that we have a responsibility to indicate to the world that we disapprove of the decision of the Medical Council to drop the matter. We should like it to be known that we dissociate ourselves, as an institution and as a body of concerned citizens and doctors, from their decision. We do this because we feel that it is morally indefensible for a matter as important and serious as this to be dropped before it has reached the Disciplinary Committee stage. We do this because we feel that the ethical standards of our profession have been compromised; because as doctors we have a humane concern for our patients and their welfare; because we are worried about the possible effects of the decision of the Medical Council on the future treatment of prisoners and detainees by the authorities; and because we are worried about the repercussions of the Medical Council's decision abroad. Already there are signs of a campaign to cut ties with doctors from South Africa, both at international congress level and in the recognition of our degrees abroad."

"Countries with which we have most dealing and reciprocity agreements, Great Britain, Australia, New Zealand, the Netherlands, Belgium, the United States of America, Canada, have always been happy to accept the *bona fides* of the S.A. Medical and Dental Council. However, there are already indications that rethinking on this point has started. The British Medical Association Ethics Committee is to examine the Biko doctors decision of the S.A. Medical and Dental Council. In Geneva, the World Health Assembly has called on member countries to review their medical ties with South African doctors."

My Faculty believes that it is vitally important that we distance ourselves from the decision of the Council. As a group of concerned doctors and Medical School teachers, we are worried that the South African Medical and Dental Council might, by its decision, have called in question its own credibility as an objective and unbiased

guardian of the high standards of the medical profession in South Africa.

Yours faithfully,

PHILLIP V. TOBIAS

Dean, Medical School,
University of Witwatersrand,
Johannesburg, S.A.

Russian continues

SIR, — May I, as Head of the Department of Russian and Soviet Studies at the University of Lancaster, be allowed to comment briefly on Vera Rich's piece in the last issue of *Nature* (3 July). Her remarks concerning the diminishing provision for the study of Russian in Britain, in contrast to the proposed expansion in Finland, are entirely justified. Her statement, however, suggesting there has been an "overnight decision to close down the Russian Department of the University of Lancaster" is incorrect and seriously misleading.

As part of a broader policy document which purported to be a strategy for the 1980s, a small Vice-Chancellorial committee at Lancaster recently recommended the gradual phasing-out of five small academic departments, including Russian. The document was subjected to widespread and vociferous criticism throughout the University and was finally withdrawn at a meeting of the University Senate on 11 June.

The department, therefore, still exists and will continue to exist — with an increased undergraduate enrolment in 1980-81. Moreover, in addition to its full degree programme of Russian history, politics, language and literary studies, and in the context of your article, it plans to mount a new elementary Russian language course which will be available for scientists who wish to acquire a reading knowledge of Russian and hence gain access to a major "medium of scientific communication".

Yours faithfully,

ALAN WOOD

University of Lancaster, UK

Dioxin disclaimer

SIR, — I wonder if you would be kind enough to allow me to comment on Alastair Hay's article in *Nature* (6 March) which indirectly implies that I had some part in the non-publication of the two investigations of Coalite workers.

Firstly I was never involved in the treatment of Coalite cases. Secondly, not only did I never suggest suppression of the data, but I repeatedly urged its publication — unsuccessfully.

It should be mentioned that the second and more far-reaching study to which Hay has referred was suggested to Coalite by the HSE since the first study was considered inadequate. I have never been shown the results of this second study by the company but obtained them by unofficial means.

Yours faithfully,

K.D. CROW

Princess Margaret Hospital
Swindon, UK

NEWS AND VIEWS

Tectonics of the Northwest European Continental shelf

from P.E. Kent

BOTH academic and industrial organisations are involved in exploration of continental shelves, but it is only intermittently that the large amount of data assembled by industry is made generally available. One of these occasions — five years from the previous major release¹ — took place at a conference held in London in February².

The conference in 1975 had been concerned mainly with the results of industrial exploration of the North Sea, at a time when drilling in the outer basins was just beginning. This year there was an increased input from other sources — notably regional compilations on the North Sea area by the Institute of Geological Sciences, and on the Norwegian offshore by the Norwegian Petroleum Directorate, while the oil companies added to North Sea data and described for the first time the structure and stratigraphy of the more westerly basins.

In the North Sea itself the nature of the crystalline floor was described from cores and radioactive dating at fifty sites. There is, as previously deduced, continuity of continental crust across the North Sea with a degree of attenuation indicated by gravity measurements and with Caledonian metamorphics with granite intrusions extending as far south as a line from Dundee to Stavanger. The area between this line and the Welsh-Brabant massif is occupied by lightly tectonised sediments, which have locally yielded Lower Palaeozoic faunas. It was again emphasised that development of the North Sea basin began in the Upper Palaeozoic, and that the Mesozoic fault pattern frequently relates to Caledonian progenitors.

Although the additions to North Sea data and detailed descriptions of additional fields are of considerable

importance, the main access of new knowledge is of the basins nearer the edge of the continental shelf, from the Barents Sea to the Bay of Biscay, outlined in the following paragraphs.

The Norwegian Shelf, terminated seaward by the Faeroe-Shetland and Voring Plateau escarpments and extending to 68°N, is occupied by a series of basins partly separated by longitudinal and transverse faulted highs. In these basins sedimentary thicknesses exceed 12 kilometres, often in highly asymmetrical trap door-type half-graben basins which ended their fault-development with the 'Late Cimmerian' phase and which were essentially growth-fault controlled. Stratigraphic horizons have been partly dated in relation to those of the adjoining North Sea, and it is deduced that the greater part of the sedimentary sequence is Mesozoic, but that basinal development began in the Carboniferous.

In contrast to the northern North Sea, but in line with other fault controlled Mesozoic basins, inversion took place during the Cretaceous.

In the Barents Sea, more remote and also still undrilled, dating of horizons is less certain and depends on a combination of local dredgings, regional and stratigraphic trends (with Svalbard as a northerly control point) and deduction from world wide events.

The area shows two sub-basins, each with large salt diapirs which originated below the presumed Permian and hence believed to be Devonian/Carboniferous. The existence of salt of this date, if substantiated by drilling, could have an important influence on our understanding of Permo-Triassic salt deposition.

West of the Shetlands the notable feature has been discovery of a very large oilfield on the Rona Ridge. This has been credited with reserves in place of 3.5–4 billion barrels (around 500 million tons), but it is a heavy oil which will present major problems in extraction. The reservoir is mainly of Carboniferous and Devonian

sandstones capping the scarp of a large down-to-the-ocean fault which bounds the main Rockall basin. The adjoining trap-door type basin on the eastern side of the Rona ridge is too shallow to have generated significant amounts of hydrocarbons, and it has to be concluded that the source rocks for this very large field lie in the deep Rockall trough itself, an area consequently considered increasingly attractive for future exploration.

The Northern Irish Sea, the site of discovery of a large gas field, is confirmed as essentially a Permo-Triassic basin analogous to the Cheshire Basin on land. An unexpected feature however is the discovery that it underwent inversion — a process of relatively rapid uplift at the end of the Mesozoic, partly counteracting the long period of Permo-Triassic subsidence, a feature now recognised widely on this continental shelf.

The Kish Bank Basin, a simple trapdoor subsidence lying off the Irish coast near Dublin, illustrates particularly well progressive basinal development from Upper Palaeozoic to Tertiary. The tectonised older Palaeozoic floor is followed by Carboniferous — possible Viséan, then Westphalian B, C and D — Permian, Triassic and Jurassic (the latest formation surviving). The Carboniferous, Lower and Upper Triassic each thicken towards the 5,000m down-throw boundary fault system, which developed as deposition continued and moved also in the post-Jurassic, finally in the Oligocene.

In Cardigan Bay a very deep Mesozoic and Tertiary basin lies alongside the seaward continuation of the NE-SW Bala fault, which has a westerly downthrow of 4,000 metres. The oldest beds identified are Upper Carboniferous; these are overlain by Permo-Triassic and Jurassic sediments which total 7,000 metres in the centre of the basin. Contrary to earlier expectation the

P.E. Kent is a former chairman of the National Environment Research Council and the Petroleum Exploration Society of Great Britain.

*The conference was held jointly by the Institute of Petroleum, the Ecological Society and the Petroleum Exploration Society of Great Britain. The Proceedings will be published by the Institute of Petroleum in 1980.

Cretaceous is absent, and the faulted earlier Mesozoic is directly overlain by Tertiary sediments. In line with the development of other Mesozoic basins on the continental shelf it could be presumed that movement of the bounding fault was syn-sedimentary, but the five deep boreholes drilled in the basin were not sufficiently close to the fault to encounter marginal fans, if these exist. The Cretaceous may have been present and been eroded, perhaps a case of inversion of subsidence as in analogous basins elsewhere around Britain.

The Celtic Sea Basin: The Celtic Sea conceals one of the larger basins of the continental shelf, extending 300 km southwards from the Irish coast. Like the other basins of the region, the Celtic Sea basin has a long history, with late Palaeozoic rocks (Lower Permian) proved at depth. Basin development until the late Mesozoic was controlled by rift development. Undifferentiated continental Permo-Triassic rocks and a largely complete Jurassic sequence ending with the Purbeck was followed by a thick development of strata in the Wealden facies (indicated as some 1,200 metres thick in the Kinsale Head field); the Wealden apparently extending through the Neocomian to early Albian — appreciably longer than in southern England.

The Kinsale Head gas-bearing structure is an anticlinal feature produced by an inversion of the central part of the basin, a process which gently arched the later Wealden and overlying beds — a close analogy to the English Weald.

The Fastnet Basin: The Fastnet Basin, an appendage of the Celtic Sea Basin extending southwestwards almost to the continental margin in Irish waters, is a fault-bounded subsidence, measuring 110 km from NE to SW and 35–40 km across, which has been investigated by gravity, aeromagnetic and repeated seismic surveys followed by nine exploration wells, unfortunately without any economic discovery. The general structural style is analogous to that of most other basins on the continental shelf, with a thick packet of earlier Mesozoic rocks affected by strong faulting which died out before or during the Upper Cretaceous. An Upper Palaeozoic floor was reached in two wells which respectively identified Lower Carboniferous (Tournaisian) and Devonian, succeeded by a 'normal' continental Permo-Triassic development and a greatly expanded Lower Jurassic. Unconformable Cretaceous begins with a Wealden episode more limited in time than that of the Celtic Sea but similar in facies and age (Berriasian) to that of southern England. It is itself unconformably transgressed by 'Greensand', Chalk and

Tertiary sediments.

The closely spaced faulting is dominantly extensional, down-to-basin, most faults being truncated by the Cretaceous unconformity, but others dying out in the Upper Cretaceous. The few which have large displacements in the Upper Cretaceous show evidence of throw reversal, this being associated with extensive basin inversion. The inversion process began in the Lower Cretaceous and resulted in attenuation of both that formation and the Chalk over the area where the Jurassic is thickest, the Chalk itself being absent over an area 20×10 km.

The listric faulting developed during the Jurassic can be seen as extension in relation to the incipient Atlantic continental parting, but the development of strong basin inversion (normally characteristic of internal basins) might be taken to indicate that the limit of the continental crust lies at least tens of miles oceanward of the shelf break.

The Bay of Biscay: The concept of the Cretaceous anti-clockwise rotation of the Iberian peninsula relative to the rest of Europe is widely accepted and the paper submitted to the 1980 Conference most usefully integrated this incident with the results of oil company exploration along the northern Spanish coast. The continental split took place centrally in a little-disturbed shelf covered by Triassic and Jurassic rocks. The preliminary stretching associated with the development of the oceanic wedge produced tension faulting and the consequent development of trapdoor subsidence, with greatly expanded Upper Jurassic and Lower

Cretaceous sedimentation in the marginal 7 km deep Spanish Trough in the south and in the shallower Armorican Trough to the north of the incipient parting. On the new evidence the split radiates not from Biarritz but 120 km further north in the Parentis basin. The Spanish Trough suffered strong compression in association with the Pyrenean orogeny (Upper Eocene) and has by some been regarded as the foredeep of a subduction zone, but if the stratigraphical datings given in this paper are confirmed the sedimentary accumulation long predated this compressional phase.

In summary, the basins on the Northwest European Continental Shelf lying north of the Hercynian orogenic belt are known in the majority of cases to have a history extending back to Carboniferous or Devonian, with the main subsidence taking place during the Permo-Triassic and Jurassic. Throughout this period the stress system seems to have been consistent and to have been relieved by tensional, often listric, faulting which may well be symptomatic of thinning of the underlying crust by a process of plastic stretching. This long taphrogenic period ended with the inception of Upper Cretaceous and Tertiary simple miogeosynclinal subsidence — a process which was however interrupted in a majority of the basins by an episode of inversion of the former downwarp. In some cases this inversion may be related to a shortlived change in shear stresses but it was apparently not limited to subsidences produced by Mesozoic faulting and is not yet fully understood. □

Special sites in genetic recombination

from John Rosamond

At the end of the 1978 Cold Spring Harbor Symposium, Frank Stahl suggested that genetic recombination was ceasing to be simply a geneticists domain, and that the time was right for a more biochemical approach. A number of people seem to have taken notice of this since considerable effort is being expended on the *in vitro* formation and analysis of intermediates in recombination. Of particular interest is the work of Stahl himself, and his collaborators at the University of Oregon, on the nature of special sites for genetic recombination. Their recent work suggests that such sites may be structurally complex (Cell, 19, 785; 1980) and of fundamental importance in recombination.

Generalised recombination involves the exchange of genetic information between homologous chromosomes, and it used to be thought that the frequency with which recombination occurred was equal throughout all regions of homology. That

this expectation is not always met was first shown by genetic studies of recombination in fungi¹. The frequency of exchange was higher in some regions of homology than others, implying the presence of special sites (hotspots) in the genome which influence the frequency of recombination in that region. More recently, Stahl and his collaborators have been examining similar sites which promote recombination in bacteriophage λ . Their studies on these sites, termed Chi, not only have provided interesting information on the mechanism of genetic recombination at the molecular level, but also suggest that the involvement of special sites, such as Chi, in genetic recombination may be more widespread than was originally envisaged.

Chi sites are not present in wild-type bacteriophage λ , but are found as mutations that arise in response to selective pressure on a specific part of the λ life cycle. After infecting *Escherichia coli*, the λ genome circularises and replicates, initially by a Cairns-type 'theta' mechanism, to produce a pool of monomeric circular molecules, before

1. Woodland, A. W. (ed.) *Petroleum and the Continental Shelf of North West Europe* Vol. 1 (Applied Science, Barking, UK, 1975).
2. Illing, I. V. (ed.) *Petroleum Geology of the Continental Shelf of North-west Europe* (Heyden, London, in the press).

John Rosamond is in the Microbiology Unit, Department of Biochemistry, University of Oxford.

switching to the 'rolling circle' mode of replication. Most progeny chromosomes are derived from the concatamers produced in this way, since the monomeric circular molecules are not a substrate for encapsidation. Transition from 'theta' to 'rolling circle' replication is facilitated by two λ genes, *red*⁺ and *gam*⁺; the *red*⁺ gene product promotes replication by creating replication forks, while the *gam*⁺ gene product inhibits a host nuclease which would otherwise prevent rolling circle replication. (This nuclease, exonuclease V, is the product of the *recB* and *recC* genes and is involved in a bacterial recombination pathway).

Consequently, when phage that are defective in these genes (λ *red gam*) are grown on *recBC*⁺ bacteria, rolling-circle replication is prevented, and in order to organise the DNA into a form that can serve as a substrate for encapsidation, the monomer genomes must be recombined to form dimeric molecules. λ *red gam*⁻ phage do this relatively inefficiently, thereby producing small numbers of progeny phage and therefore small plaques.

In these circumstances, there are obvious advantages to be gained by increasing the efficiency with which the host recombination system is being used. Consequently, when λ *red gam*⁻ phage are repeatedly grown on a *recBC*⁺ host, the small-plaque forming phage are eventually overgrown by mutants with a large plaque phenotype². This is the Chi⁺ phenotype,

which arises after mutation in one of at least four loci dispersed throughout the λ genome. It was suggested that these mutations (χ ⁺) increased the yield of progeny phage by accelerating the rate limiting step in the recombination pathway being used to form the dimeric precursors of mature phage DNA; it is the nature and properties of these χ ⁺ mutations that Stahl and co-workers have been investigating.

The presence of a χ ⁺ mutation in the λ genome increases the frequency of recombination near the mutation by about 10-fold. This stimulation decreases with distance, but can still be detected up to 2×10^4 base pairs away from Chi. There are four recombination pathways open to λ , and, in principle, Chi⁺ might have enhanced recombination mediated by any or all of these. It transpired, though, that the action of Chi⁺ is specific to the major bacterial recombination system, the so-called *recBC* pathway, which required the product of the *recA* gene as well as those of *recB* and *recC*³. This discovery led to the suggestion that Chi sites occurred in the *E. coli* genome as well as in λ , and functioned in bacterial recombination. Indeed it was found that λ *red gam*⁻ which include the *E. coli bio* operon behaved as though they carried a Chi⁺ site, since they made large plaques on *recB*⁺ hosts⁴. This property was not peculiar to the *bio* operon though, since by cleaving *E. coli* DNA with *EcoRI* and inserting random fragments into the λ genome, Malone *et al.*⁵ estimated that Chi

hotspots were present naturally in the *E. coli* chromosome at a density of 1-2 per 10^4 base pairs. Similar experiments, in which *EcoRI* fragments of DNA from *Saccharomyces cerevisiae* were inserted into λ , showed that yeast DNA also carries Chi sites⁶.

Meanwhile, the phenotype of the χ ⁺ mutations in λ had been investigated in more detail and it was found that the enhancement of recombination due to Chi⁺ was asymmetric (*Mol. Gen. Genet.* **140**, 29; 1975). Thus, χ ⁺C in the λ *cII* gene stimulated recombination to a greater extent in the *J-N* region to the left of χ ⁺C than it did in the *P-R* region to the right, although χ ⁺C was closer to the *P-R* interval. This leftward bias of Chi⁺ activity was common not only to all the Chi⁺ sites arising by mutation in λ , but also to all the naturally occurring Chi⁺ sites found on the *EcoRI* fragments of *E. coli* DNA⁶.

Of the several possibilities for this directional bias of Chi activity, two are most likely; either Chi could itself be an asymmetric sequence, in which case the directionality of Chi⁺ activity will be dependent on the orientation of the sequence, or else the directionality could be imposed by some aspect of phage λ biology. If the directional bias were due solely to the orientation of the Chi elements, then artificially reversing the Chi sequence should lead to a reversal of the directional bias. Experiments to test this were done by excising *chi*⁺-carrying *EcoRI* fragments of *E. coli* DNA from λ *red gam*⁻ phage and



100 years ago

A sad balloon accident has taken place at Le Mans, and may be referred to as illustrating some useful facts relating to aeronautics. A man named Petit had ascended with two balloons connected by a long rope. The smaller, which was placed above, carried his son, almost a boy; Petit being in the larger with his wife. There was not much wind, and this foolish experiment would have ended without accident if Petit had not forgotten to loosen the neck of the balloon, so that no escape was left for the gas which was gradually expanding. When the balloon arrived at an altitude of 400 to 500 metres it burst in the vicinity of the "equator," and descended with great velocity, dragging the smaller balloon. Petit, who was devoid of any scientific knowledge, supposed his son was in danger, and with true heroism he cut the rope connecting the two balloons when at 250 to 300 metres from the ground. He placed his wife on the ring and remained himself in the car. The shock was so terrible that his spinal cord was broken, and he died

on the following day. His wife was very badly hurt, and though in danger, is alive. If Petit had not cut the rope by an act of unintelligent devotion he would very likely have escaped, and his son would not even have touched the ground.

Through the kindness of General Myer we have received some further details concerning the extraordinary memory credited to the man in charge of the hat room at the Fifth Avenue Hotel, New York, referred to in *Nature*, vol. xxi. p. 562. He is an Irish-American about thirty years of age, Gilmartin by name, and has occupied his present position about a year. His sole duty consists in looking after hats during meal hours. The fact of his possessing a remarkable memory is indisputable, but still he is not looked upon as a prodigy by the hotel officials. They state that a Tommy Hart, now dead, who figured conspicuously in the "Stoke trial," was for a long time in charge of their hat-room, and was this man's superior as regards memory, and cited other instances of men now employed in different hotels throughout the country whom they consider his equals. It is very evident, however, that he possesses a wonderful talent for selecting the right hats, and mistakes are rare with him.

An occurrence, which may be partially or wholly attributable to the earthquake which Switzerland has recently undergone, is reported by the *Times* Geneva correspondent from Quarten, in the canton

of St. Gall. A short time ago the people of the neighbourhood noticed signs of uneasiness about the Schnebelberg. The summit of the mountain appeared to be in a very precarious position, and it was feared that it might slip down and overwhelm the Schnebelwald, an extensive wood in the valley below. In anticipation of a possible catastrophe, great efforts were made to cut down and carry away as many trees as possible, though the men engaged in the work wrought at the peril of their lives. On Sunday fortnight, when fortunately there was nobody in the wood, a deafening report, like the firing of heavy artillery, resounded through the valley, and the mountain was hidden from view by a thick cloud of dust. When it dispersed, the Schnebelberg was seen to be shorter by a few metres, and the beautiful wood in the Murgthal has disappeared beneath a huge avalanche of stones and earth.

News from the Azores states that a disturbance of the earth has occurred in the island of St. George, resulting in the formation of another small island of about 18,000 square yards, and distant 600 yards from the shore.

It is stated that Col. Prjevalsky and his party are prisoners in the hands of the Chinese, who, it will be remembered, prevented him from proceeding to Lhasa.

From *Nature* 22, 15 July, 252-254, 1880.

re-inserting them in the opposite orientation⁷. After inversion, some fragments that had been Chi⁺ become Chi⁻ and some Chi⁻ became Chi⁺, while others showed no change in their phenotype. In all cases, though, the Chi⁺ phenotype showed the original leftward bias in stimulation of recombination. Clearly, reversing the Chi element does not lead to a reversal of the directional bias, which implies that some aspect of this bias is a property of λ and not of Chi. Nonetheless, the fact that the Chi⁺ phenotype is orientation dependent suggests that the Chi sites themselves are asymmetric nucleotide sequences.

Direct proof of this has come from the work of Gerry Smith and colleagues at Oregon^{8,9}. They found the nucleotide sequence around the χ^+ mutation in λ *cII* (χ^+C) was strikingly devoid of any unusual structural features, such as obvious symmetrical regions, though they were unable, having determined the sequence of a single χ^+ locus, to deduce the boundaries of the Chi site.

More recently, they have sequenced a second Chi site in λ (χ^+B , lying between *xis* and *redA*); comparison of this sequence with χ^+C sequence reveals a surprising extent of homology¹⁰. Within a region of 30 base pairs, there are 23 base pairs common to both sequences. The largest uninterrupted common sequence is 9 base pairs, with another common sequence of 6 base pairs to its left. Not only is the sequence asymmetric, but it may be that the nucleotide sequence of Chi sites is hyphenated, with regions of random base sequence separating specific nucleotide sequences. Such nucleotide sequences are found at the recognition sites of the Type I restriction endonucleases, and here too the asymmetry of the recognition site is reflected in the asymmetry of the restriction enzyme activity¹¹. Again, though, the extent of the common sequence essential for the Chi⁺ phenotype cannot be identified; this will require the determination of the nucleotide sequence both of other Chi sites, particularly the *chi*⁺ loci that occur naturally in *E. coli*, and of *chi*⁻ revertants. Given the frequency with which Chi sites occur in the *E. coli* genome, if subsequent work shows them to

have a complex, hyphenated sequence, this finding would argue both for a strong selective pressure for the maintenance of these sites and for their having a fundamentally important function. At present, although we know that Chi sites in *E. coli* DNA are capable of stimulating recombination when introduced into the λ genome, evidence that these sites perform a similar function *in situ* is circumstantial, at best.

Another major consideration is the mechanism by which Chi acts and the implications of that mechanism (be it confined to λ , or of more widespread application). Since, as noted above, we know that the stimulation of recombination by Chi⁺ is specific for the so-called *recBC* pathway, the underlying assumption is that Chi⁺ is recognised by a protein, so as to accelerate a rate-limiting step in the recombination process. The *recBC* pathway requires the activity of proteins coded by the *recA*, *recB* and *recC* genes (and possibly the *lexC* gene product as well); it remains possible that other, as yet unidentified, proteins are involved. We now think that the *recA* protein is involved in one or more of the early events in recom-

bination, and probably promotes the formation of heteroduplex molecules¹². We are less certain when (or how) *recBC* protein acts, although there is genetic evidence to suggest that it is involved late in recombination, perhaps in the resolution of heteroduplex DNA to form recombinant molecules¹³. Since it is thought that Chi also acts late in the *recBC* pathway¹³ it seems more likely that the Chi nucleotide sequence is recognised by the *recBC* gene product than by the *recA* gene product. Disappointingly, efforts to show a specific interaction between Chi⁺ sequences and the *recBC* enzyme have been unsuccessful, although the problem is complicated by the diversity of the activities and mechanisms of the *recBC* enzyme^{14,15}.

Nonetheless, Stahl has offered a working hypothesis to describe the mechanism of Chi-stimulated recombination in phage λ , a component of which is that Chi is a specific site for the nucleolytic activity of the *recBC* enzyme¹⁶. Whether this idea is correct, and what its application might be to genetic recombination in other organisms, remains to be determined. □

The structure of zeolites

from L. V. C. Rees

ZEOLITES are silicates of sodium, potassium or calcium. There are two varieties of zeolites, with three dimensional cage-like lattice or open channel structure that allow zeolites to be used as 'molecular sieves'. The high-resolution electron microscope pictures of zeolites described in the previous issue of *Nature* (286, 111; 1980) were first revealed when a few early results were shown at two recent meetings.* The excitement they caused there undoubtedly arose from the ability of zeolite chemists to actually see the windows in the aluminosilicate crystals and not to be entirely dependent on the subjective interpretations of X-ray crystallographers.

Some of the most heated discussions took place among the small group of X-ray crystallographers who argued about the existence or otherwise of zero-coordinated cations in some zeolite structures and about the quality of the data presented by the respective X-ray groups². The lack of large single crystals of zeolites was also bemoaned. It is, therefore, somewhat refreshing to actually observe the unit cells in these high-resolution microscope photographs and to find that Thomas and colleagues had to grind the 10-50 μ m crystals down to smaller dimensions before they could be studied in the microscope!

Not only can one now observe the apertures in these structures but we should be able to see the cations sitting in these windows when the resolution of the microscope is improved to 2.4 Å and when zeolites which are known to contain cations in the apertures after dehydration are studied (for example, potassium form zeolite A).

High-resolution electron microscopy should also be of great help to zeolite chemists. Zeolites, when used as catalysts by the petroleum industry for the cracking of hydrocarbons, require to be frequently decoked at high temperatures. Much effort has gone therefore, in producing zeolites which are more thermally stable. The rates of vitrification in the electron beam described in the *Nature* paper by Bursill *et al.* should, hopefully, provide information on some of the factors which affect the thermal stability of zeolites. A method of treating zeolite Y to produce thermally, ultra-stable zeolite Y has been developed³. In this treatment aluminium ions are removed from the framework and deposited in the zeolite channels as hydroxoaluminium cations. Much doubt still exists as to the exact nature of these aluminium products and on the effect of the loss of the framework aluminium on

1. Murray, N.E. *Genetics* **48**, 1163 (1963).
2. Henderson, D. & Weil, J. *Genetics* **79**, 143 (1975).
3. Stahl, F.W. & Stahl, M.M. *Genetics* **86**, 715 (1977).
4. Malone, R.E. & Chatteraj, D.K. *Mol. Gen. Genet.* **143**, 35 (1975).
5. Malone, R.E. *et al.* *J. Mol. Biol.* **121**, 473 (1978).
6. Chatteraj, D.K. *et al.* *Cold Spring Harbor Symp. Quant. Biol.* **43**, 1063 (1978).
7. Faulds, D. *et al.* *J. Mol. Biol.* **131**, 681 (1979).
8. Smith, G.R., Faulds, D.H. & Sprague, K.U. *Cold Spring Harbor Symp. Quant. Biol.* **43**, 1067 (1978).
9. Sprague, K.U., Faulds, D.H. & Smith G.R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6182 (1978).
10. Smith, G.R., Schultz, D.W. & Crasemann, J.M. *Cell* **19**, 785 (1980).
11. Rosamond, J., Endlich, B. & Linn, S. *J. Mol. Biol.* **129**, 619 (1979).
12. McEntee, K., Weinstock, G.M. & Lehman, I.R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2615 (1979).
13. Birge, E.A. & Low, K.B. *J. Mol. Biol.* **83**, 447 (1974).
14. Rosamond, J., Telfander, K.M. & Linn, S. *J. Biol. Chem.* **254**, 8646 (1979).
15. Telfander-Muskovitch, K.M. & Linn, S. *ICN-UCLA Symposia on Molecular and Cellular Biology* xix in the press.
16. Stahl, F.W. *Ann. Rev. Genet.* **13**, 7 (1979).

L. V. C. Rees is in the Department of Chemistry, Imperial College of Science and Technology, London.

*The British Zeolite Association met in Thornaby-on-Tees, 12-13 April, 1980 and the Fifth International Zeolite Meeting was held in Naples, 2-6 June, 1980.

the crystallinity of the zeolite. High-resolution electron microscopy could possibly observe these hydroxoaluminium cations and, along with electron diffraction, the structure of the resulting framework could be ascertained.

Bifunctional zeolite catalysts are widely used for hydrogenation-dehydrogenation reactions. These catalysts contain the usual Bronsted and Lewis acid sites necessary to produce carbonium ions but also contain atomic platinum or other metallic elements in the zeolitic cavities. These metal atoms often diffuse from the cavities and aggregate on the surface of the zeolite crystals and the catalyst loses much of its effectiveness. High-resolution electron microscopy would be a useful tool to ascertain the factors affecting this aggregation process, provided the specimens display sufficient beam stability for adequate imaging to be performed.

Natural mordenites and synthetic mordenites are found to give the same X-ray diffraction patterns. From these X-ray studies the structure of mordenite has been determined. This zeolite has a main elliptical channel, running parallel with the *c* axis of dimensions 0.67×0.70 nm. Natural mordenite, referred to as

small-port mordenite, has an effective diameter of channel of ~ 0.4 nm while synthetic mordenite, called large-port mordenite has channels consistent with the proposed structure.

The adsorbate molecules which can enter the natural mordenite channels are much smaller than the molecular which can be adsorbed in the synthetic mordenites. It has been suggested that a few fault planes in the natural mordenites would produce these results in this zeolite with a one-directional channel system. However doubts still remain as to the exact reason for this difference and it would be interesting to look at these two different forms using high-resolution electron microscopy.

The paper of Bursill *et al.* presents evidence of $\text{Si}^{4+}/\text{Al}^{3+}$ ordering. This is

another fascinating result of their studies. In a recent "Workshop on the Adsorption of Hydrocarbons in Zeolites" held in Berlin, Aldershaft from 19-22 November, 1979 Engelhardt *et al.* presented exciting results obtained by high resolution, magic-angle ^{29}Si n.m.r. which suggested that in a zeolite A which had an exact 1:1 ratio of Si:Al the majority of the Si ions in the framework were bonded through oxygens to three Al and one Si ions. Thus some Al-O-Al bonding must exist in this zeolite and Loewenstein's rule is not obeyed. Other confirmation of these findings can be found in the literature, for example four of the X-ray reflections in the paper by Pluth and Smith⁴ do not comply with the Fm3c space-group proposed by these authors but are consistent with the Fm3 space-group which would exist if Si/Al ordering occurred in their zeolite A sample⁵.

In the past many chemical analyses of zeolite A have found excess Al to be present in the material. To abide by Loewenstein's rule Barrer and Meier proposed⁶ that some NaAlO_2 was occluded in the sodalite cages. However these new results suggest that this conclusion may not be necessary and we await further studies with great anticipation. □

1. Bursill, L.A., Lodge, E.A. & Thomas, J.M. *Nature* **286**, 111 (1980).
2. Smith, J.V. *Proceedings of the Fifth International Conference on Zeolites* **194**, (ed. Rees, L.V.C., Heyden, London 1980).
3. McDaniel C.V. & Maher P.K. *Molecular Sieves* **186**, (Society of Chemical Industry, London 1968).
4. Pluth, J.L. & Smith, J.V. *J. Phys. Chem.* **83**, 741 (1979).
5. Lodge, E.A., Bursill, L.A. & Thomas J.M. in preparation.
6. Barrer, R.M. & Meier, W.M. *Trans. Faraday Soc.* **54**, 1074 (1958).

Epidermal growth factor and mitogenesis

from Colin R. Hopkins

FOR exploring the interactions of hormones with their target cells epidermal growth factor (EGF) is an extraordinarily versatile probe. It can be loaded with radioactive, fluorescent, photoaffinity and even particulate labels without losing its ability to find and follow its receptor. Because, in addition, it provokes a mitogenic response in the most widely used fibroblast cell lines EGF is currently the favourite of all labelled ligands.

The receptors for labelled EGF on cells cultured in low serum are of one class and randomly distributed¹⁻⁶. On binding the peptide, the receptors aggregate and are then taken into the cell by endocytosis. The number and size of the receptor aggregates and the pattern of redistribution varies considerably in different systems and it is not yet clear if this is due to differences in the various kinds of EGF probe or to real differences in cell surface behaviour. Certainly the relationship between the degree of receptor occupation, the rate of receptor internalization and the strength of mitogenic stimulus is very complex. Thus, although only one class of EGF receptors appears to exist on most cells⁷ several studies have shown that only a small

proportion of the total population needs to be occupied for the maximum mitogenic response to be generated^{8,9}. At low concentrations of peptide the rate of receptor internalization correlates well with mitogenic potency, but at higher concentrations internalization continues to increase proportionally but mitogenicity is much reduced¹⁰.

Clathrin-coated pits, which play such a prominent role in low density lipo-protein uptake¹¹, play an important³ and even exclusive¹² role in internalization of EGF in some cells whereas in others⁵ uncoated pits seem to predominate. If coat components are found to play a part in either receptor attachment¹⁴, or intracellular routing¹⁵, then the differences may be functionally very important, perhaps relating to the selection of alternative intracellular destinations.

Ferritin-tagged EGF allows the identification of receptor complexes by electron microscopy¹⁶. After uptake, the complexes remain associated with the limiting membrane of the endocytosed vesicle until it becomes transformed into a multivesicular body (MVB). The MVB forms when receptor-rich vesicles are pinched off from the limiting membrane of the vesicle into its internum. Within about 30 minutes the receptor-bearing vesicles

disappear and the MVB transforms into a secondary lysosome containing free ferritin particles. This scheme neatly accounts for the earlier work of Das and Fox⁹ who showed that the complete membrane bound EGF-receptor complex, and not just the EGF peptide, is degraded in the lysosome.

As the path of the EGF-receptor complex has been followed as far as the point of degradation, the crucial question is which step gives rise to the second messenger(s) responsible for mitogenesis. Is there a membrane based receptor-transducer mechanism acting near the beginning of the pathway, or is there a lysosomal 'activation mechanism' generating active fragments (cf Diphtheria toxin¹⁷) acting near the end? Chemical modulations of the EGF-induced mitogenic response may help to choose between these alternatives although the most intriguing effects are induced by agents like amine compounds and colchicine, which have ill defined sites of action.

Among the more specific effects of these early stage modulators of EGF mitogenicity are those induced by phorbol esters^{18,19} and glucocorticoids²⁰, which have been shown to alter the affinity of EGF receptors, and retinoids, which selectively increase the number of EGF

Colin R. Hopkins is Professor of Cell Biology at the University of Liverpool.

receptors available²¹. The importance of receptor aggregation has been indicated by the finding that cyanogen bromide-treated EGF which binds, but is poorly mitogenic, can be made considerably more potent when crosslinked with EGF antibody²². Here, the most straightforward interpretation — that potency is related to the number of occupied receptors able to aggregate and internalize — holds.

The effects of methylamine and colchicine, both of which enhance the potency of EGF without affecting the initial EGF-receptor interaction^{23,24} are open to several interpretations. Methylamine seems capable of acting at two sites at least, since it has been shown to prevent the patching of rhodamine-labelled EGF (and prevent clustering into coated pits)²⁵ as well as reducing the rate at which EGF is degraded^{26,27}. Studies using ferritin-labelled EGF show that concentrations of methylamine (10–30mM) which prevent visible patching of the fluorescent label allow some internalization of the EGF-receptor complex to occur¹⁶. It cannot be concluded, therefore, that the increase in EGF mitogenicity caused by methylamine is due solely to the prevention of internalization.

Ammonium compounds and amines have been shown to reduce EGF degradation so the potentiation of the mitogenic response seen with methylamine may be partly due to its action at the

lysosomal level. The possibility that this is mediated by the inhibition of lysosomal hydrolase²⁸ has been removed by a study²⁹ in which leupeptin, the specific cathepsin B inhibitor, was used to inhibit EGF breakdown. While leupeptin retarded degradation so that EGF accumulated intracellularly the mitogenic response still correlated with the concentration of peptide in the medium. The effect of methylamine must, therefore, occur before the breakdown of the EGF-receptor complex. As an effect of amines is to reduce the formation of receptor-bearing vesicles within the MVB,¹⁶ the vesiculation of receptor-rich membrane, either at the prelysosome stage, or at the plasma membrane seems a likely site for amine action.

A general mechanism, proposed by Maxfield²⁵ and Davies,³⁰ whereby receptor aggregation induced by monovalent ligands may be inhibited by agents interfering with transglutaminase, an enzyme capable of cross-linking adjacent glutamyl and lysyl residues, may explain the effects of methylamine. Methylamine is a strong inhibitor of transglutaminase at concentrations which inhibit patching of rhodamine labelled EGF²³ and as it readily

penetrates cell membranes, it may act in a similar way at intracellular sites too.

These recent studies clearly show that the intact EGF-receptor complex is responsible for transmitting the mitogenic signal. The site of transduction is unclear, but the correlation between a reduction in the rate of degradation and enhanced potency suggests that the dismantling of the EGF-receptor complex and the termination of transduction are closely linked²⁴.

The role of endocytosis in the processing of the EGF-receptor complex remains unsolved. For the full mitogenic potency of EGF to be expressed, there is growing evidence that the target cell must be exposed to the peptide for periods of several hours^{8,10,29,33} and thus the rapid rate at which receptor complexes are dispatched from the surface is particularly difficult to understand. All options must remain open, since at the present time there is no compelling reason why internalization should be regarded as an integral part of the transduction mechanism. Indeed, the possibility that signalling by the EGF receptor complex begins with aggregation on the cell surface and then continues throughout the lifetime of the complex is clearly worthy of serious consideration. □

The fenlands

from Peter Moore

THE fenlands of East Anglia have long provided a source of energy, in the form of peat, and a rich agricultural resource for the inhabitants of the area. Darby (*Medieval Fenland*, Cambridge University Press; 1940) collated much information concerning the exploitation of these lands and shows that as early as the 13th century peat was being cut for fuel in the fens. Drainage of the fens for agricultural development first became extensive in the 17th century, but it was not until the mid 19th century that the immense engineering problems involved in drainage were efficiently tackled. The installation of pumping machinery at that time led to permanent lowering of water tables and the full agricultural potential of the fens could be realized.

One consequence of drainage, which is evident even by casual observation, is the lowering of the land surface which has resulted from 'peat wastage'. Much of fenland now lies between -1.5 and -2.5m below Ordnance Datum, which means that the maintenance of low water tables in the soils requires the pumping of water into elevated drainage channels and rivers on its way to the sea. Despite the obvious nature of this development, however, there are no precise records of the rate of fall in the land surface, nor of the influence of various land use practices upon this rate.

The process of peat wastage has resulted from the gradual oxidation of surface layers as microbial activity has been stimulated by the lowering of soil water tables and the consequent removal of anoxic conditions. In addition, the peat has contracted in its structure as a result of drying and some has been lost by wind erosion, combustion and removal by man. Land use practices will have played an important part in influencing the rate of these processes.

Around 1848 (the precise date not certain) a cast iron post was sunk through the peat and embedded on wooden piles in the underlying clay at Holme Fen, 10 km south of Peterborough, and the history of this post has been the subject of research by Hutchinson (*J. Ecol.* 68, 229; 1980), himself a civil engineer. When first established, the top of the post was flush with the peat surface and since that time many scattered observations and measurements have been conducted upon its progressive protrusion as the land surface fell. Hutchinson has collated these data and related them to records of the installation of new pumping machinery at various times and to variations in land use in the area.

The first drainage resulted from the

Peter Moore is in the Department of Botany, King's College, London.

- Schlessinger, T., Schechter, Y., Willingham, M.C. & Pastan, I. *Proc. natn. Acad. Sci. U.S.A.* 75, 2659 (1978).
- Haigler, H., Ash, J.F., Singer, S.J. & Cohen, S. *Proc. natn. Acad. Sci. U.S.A.* 75, 3317 (1978).
- Gorden, P., Carpentier, J., Cohen, S. & Orci, L. *Proc. natn. Acad. Sci. U.S.A.* 75, 5025 (1978).
- Maxfield, F.R., Schlessinger, J., Schechter, J., Pastan, I. & Willingham, M.C. *Cell* 14, 805 (1978).
- Haigler, H.T., McKanna, J.A. & Cohen, S. *J. Cell Biol.* 81, 382 (1979).
- Hopkins, C.R., Boothroyd, B. & Gregory, H. *J. Cell Biol.* 83, 233a (1979).
- Carpenter, G. & Cohen, S. *A. Rev. Biochem.* 10, 194 (1979).
- Schechter, Y., Harnaez, I. & Cuatrecasas, P. *Proc. natn. Acad. Sci. U.S.A.* 75, 5788 (1978).
- Das, M. & Fox, C.F. *Proc. natn. Acad. Sci. U.S.A.* 75, 2644 (1978).
- Fox, C.F. & Das, M. *J. supramolec. Struct.* 10, 595 (1979).
- Anderson, R.G.W., Brown, M.S. & Goldstein, J.L. *Cell* 10, 351 (1977).
- Willingham, M.C., Maxfield, F.R. & Pastan, I.H. *J. Cell Biol.* 82, 614 (1979).
- Heuser, J. *J. Cell Biol.* 84, 560 (1980).
- Goldstein, J.L., Anderson, R.G.W. & Brown, M.S. *Nature* 279, 679 (1979).
- Rothman, J.E. & Fine, R.F. *Proc. natn. Acad. Sci. U.S.A.* 77, 780 (1980).
- McKanna, J.A., Haigler, H.T. & Cohen, S. *Proc. natn. Acad. Sci. U.S.A.* 76, 5689 (1979).
- Pappenheimer, S. & Gill, J. *Science* 182, 353 (1973).
- Lee, F. & Weinstein, S. *Science* 202, 213 (1979).
- Brown, K.D. *Biochem. biophys. Res. Commun.* 86, 1037, (1979).
- Baker, J.B. & Cunningham, D.D. *J. supramolec. Struct.* 9, 69, (1978).
- Jentzen, A.M. *Nature* 284, 626 (1980).
- Schechter, Y., Harnaez, I., Schlessinger, J. & Cuatrecasas, P. *Nature* 278, 835 (1979).
- Maxfield, F.R., Davies, P.J.A., Klempner, I., Willingham, M.C. & Pastan, I. *Proc. natn. Acad. Sci. U.S.A.* 76, 5731 (1979).
- Brown, K.D., Friedkin, M. & Rozenzweig, E. *Proc. natn. Acad. Sci. U.S.A.* 77, 480 (1980).
- Maxfield, F.R., Willingham, M.C., Davies, P.J.A. & Pastan, I. *Nature* 277, 661 (1979).
- Carpenter, G. & Cohen, S. *J. Cell Biol.* 71, 159 (1976).
- Brown, K.D. *Cell Biol. Int. Rep.* in the press.
- Ohkuma, R. & Poole B. *Proc. natn. Acad. Sci. U.S.A.* 75, 3327 (1978).
- Savion, N., Vlodavsky, I. & Gospodarowicz, D. *Proc. natn. Acad. Sci. U.S.A.* 77, 1466 (1980).
- Davies, P.J.A., Davies, D.R., Levitzki, A., Maxfield, F.R., Milhaud, P., Willingham, M.C. & Pastan, I.H. *Nature* 283, 162 (1980).
- Ostlund, R.E., Pflieger B. & Shonfeld, G. *J. clin. Invest.* 63, 75 (1979).
- Carpenter, G. & Cohen, S. *J. Cell Physiol.* 88, 227 (1976).

construction of a channel in 1850. Within eight years, this gravity drainage system had produced a fall in the peat surface of almost 2m. A suction pump was then added, resulting in a further 0.7m fall over the next 20 years. This pumping was accompanied by the development of arable agriculture, which may have accelerated the surface lowering. A second pump, introduced in 1877 resulted in another rapid fall of a further 0.5m over the next ten years.

Agricultural depression then led to the abandonment of cereal growing over the site and the area was left as rough grazing and scrub game cover. No further lowering appears to have taken place until a further pump was introduced in 1920, but by this time a birch woodland had developed and, despite yet another pump being added in 1962, the total surface fall over the past century has been only about 0.7m.

The overall conclusions, therefore, are

that the greater part of the fall in surface level occurred immediately after the introduction of drainage and can be accounted for simply on the basis of peat contraction as it dried, rather than by any loss of organic material. In the later stages of drainage, however, lowering was more gradual and was less closely related to improved drainage; here oxidation may have begun to assume the major role.

Of particular interest is the relationship between land use and shrinkage under constant drainage processes. The replacement of arable by pastoral farming resulted in a considerable deceleration of wastage. On the basis of this observation, Hutchinson questions the wisdom of the current emphasis upon arable farming in the fens. The conservation of these organic soils could, he claims, be more effectively achieved by keeping water extraction to the minimum acceptable level and by changing the agricultural emphasis to pasture. □

al. as 0.5 or 1.0 km s⁻¹. Though uncertain, it's clear the velocity is well above the 0.25 km s⁻¹ corresponding to ice subliming into vacuum at about 200 K. Arpigny's spectroscopic studies of CH confirm enhanced velocities (0.7–1.3 km s⁻¹) and also show that the temperature of the colliding agent is several hundred K. Clearly, cometary comas do not expand adiabatically into space, but are heated probably by the kinetic energy of fragments from chemical dissociation, such as photo-dissociation of H₂O releasing 2 eV per molecule (Shul'man *Dinamika kometnikh atmosfer* §3.3; Kiev, 1972; Wallis, *Mon. Not. R. astr. Soc.* **166**, 181; 1974). This heat source more than compensates for adiabatic cooling and energy loss through rotational excitations of H₂O and other molecules in the bulk of the coma (Shimizu *Astrophys. Space Sci.* **40**, 149; 1976). The chemical energy would go primarily to H-atoms, being the light dissociation fragment. Its transfer to the heavier molecules, such as OH and H₂O is effected through collisions. Larger comets with larger collisional zones would have higher expansion velocities — if allowed for, this effect would increase the surprisingly narrow spread in derived outgassing rates.

Because the apertures of the IUE spectrographs were relatively small (4.5 × 9 × 10³ km at the comet), the variation in intensity of the intense (O, O) band of OH out through the coma could be followed. Assuming velocities and transformation rates for the parent H₂O and the OH then allows deduction of the absolute production rates. For simplicity Feldman *et al.* use the Haser-Mokhnach model describing the transformation under steady radial expansion, but take the OH to have the 1.15 km s⁻¹ velocity from photo-dissociation which should of course be isotropic in the local expansion frame. At large radial distances this simplification is acceptable (Wallis in *Cometary Missions Remeis-Sternwarte Bamberg* **12** (132), 97; 1979) but its use in the region of OH production (3–10 × 10⁴ km) means the result is rather uncertain. Unfortunately, as the H Ly α coma is optically thick in the central region, no comparable rate from the H production is derived. The only comet for which simultaneous H and OH results are available over an extended period is Comet Tago-Sato-Kosaka (Keller and Lillie *Astr. Astrophys.* **62**, 143, 1978). The aperture used there was much larger (2 × 10⁵ km at the comet) and the 26 observations show two notable characteristics: the derived OH production dropped twice as fast as H during the first three days (comet moving from 0.78–0.84 AU) and the ratio of OH:H production over most of the observation period was 0.3 rather than the 0.5 expected if H₂O was dominant. As the authors commented, the uncertainty of the ratio is large, so future comparative results from Comet Bradfield will be of great interest.

Cometary science

from M.K. Wallis

OBSERVATIONS of comets in the ultraviolet spectral region show great promise because most major components of the cometary coma (atmosphere) can be covered, instead of only dust and minor radicals such as CN and C₂.

Early in 1970, the first UV observations revealed huge atomic hydrogen comas, 15 × 10⁶ km diameter in comet Bennett, fluorescing in H Ly α (1,216 Å). During the same period, observations at 1,304 Å and 3,085 Å showed that comas of atomic oxygen and the hydroxyl radical, though less extensive, were of comparable density. The relative proportions suggested H₂O as the unobserved parent molecule — and was seen as confirming the Whipple hypothesis of comets being 'dirty' ice conglomerates.

Comet Bradfield discovered last Christmas day, just after perihelion at 0.7 AU, is favourably placed for observation from the International Ultraviolet Explorer (IUE) satellite: Feldman *et al.* (*Nature* **286**, 132; 1980) have reported the first of a very promising series of results. Optically the comet appears small and unexciting, containing little dust and hardly a trace of an ion tail. But in the UV, it is remarkably similar to the two others, Comets West and Seargent, observed in this range (1,200–3,200 Å) where H, O, CO, C, S, CS, OH, CO⁺, CO₂⁺ are identified. A high resolution scan of the OH (O, O) band is shown, the relative intensities of the components implying that the excitation is simply resonant fluorescent. Doubtless, a future detailed study will reveal some departure from purely resonant equili-

brium, due to collisional disturbances of the populations as discovered in pioneering Liège studies of CN and CH bands (reviewed by Arpigny in *The Study of Comets NASA SP-393*, 797; 1976). Such investigations are delicate, requiring allowance for doppler shifts across a solar spectrum of high resolution. But with appropriate modelling they can give parameters hardly accessible except by a cometary space-probe, namely, the OH expansion velocity and density temperature of the colliding agents (H and e).

For inferring the rate of sublimation of H₂O from Comet Bradfield, there are some uncertainties, particularly those due to the expansion velocities of different components. Past authors have assumed different values and generally normalized on H rather than OH (Keller & Lillie, *Astr. Astrophys.* **62**, 143, 1978). After adjustment, the result for the new comet comes surprisingly similar to previous comets — less H₂O by a factor of 2–4, and only three times less than Comet Tago-Sato-Kosaka, a much more active and typically impressive comet. Given that outgassing from a single icy nucleus should vary as the surface area, it's rather suspicious that now five comets, adjusted to the same heliocentric distance, should have such similar rates of loss of H₂O.

The velocity of expansion of the inferred H₂O is unknown but taken by Feldman *et*

M. K. Wallis is in the Department of Applied Mathematics and Astronomy, University College, Cardiff.

Delsemme and Combi (*Astrophys. J.* **228**, 330, 1979) also suggested recently on the basis of their comet Bennett optical observations of the 'forbidden' oxygen transition [O I] 6,300 Å, that most of it cannot arise from H₂O or OH — perhaps photo-dissociating CO₂ produces the excited atoms O(¹D), but dissociative recombination of CO⁺ could be an alternative source. However, CO⁺ is found to be very weak in comet Bradfield's UV and optical spectra. Comet Bradfield's 'trans-auroral' line [O I] 2,972 Å detected by IUE is tentatively attributed to photo-dissociation of H₂O, but CO₂ or OH are more plausible; alternatively dissociative recombinations of CO₂⁺ or OH⁺ are energetically capable of giving the O(¹S).

Some years ago Vanysek and Wickra-

masinghe (*Astrophys. Space Sci.* **33**, L19; 1975) proposed that cometary particulates could be H₂CO polymers, this being more plausible on abundance grounds than the commonly assumed silicate grains. For interstellar grains, it seems now to be clear from the 3µm absorption feature that there can only be a small proportion of H₂O-ice, while general abundance arguments imply an organic composition (Whittet *Nature* **281**, 708; 1979; Hoyle & Wickramasinghe *Astrophys. Space Sci.* **69**, 511; 1980). So cometary scientists need to consider more carefully whether H₂O-ice really does constitute a major fraction of comet nuclei — the continuing IUE observations of Comet Bradfield could lead to a resolution of this fundamental issue. □

cell lines that had been transformed *in vitro* and found to be tumourigenic only in immune-deprived (thymectomised, irradiated, foetal liver restored) mice, he found that these lines were also NK sensitive. However, after passage through the immune deprived mice, the cell lines were tumourigenic in normal mice, and these tumours were now NK resistant. The immune deprived mice are deficient in NK activity at the time of tumour inoculation, but by 2-3 months these mice have half-normal levels of NK. The inability of these *in vitro* derived cell lines to grow as tumours in normal mice is closely correlated with acquired NK resistance. Reconstitution experiments in this system are in progress and may provide the best evidence to date that NK activity is indeed relevant to early surveillance against incipient tumours *in vivo*.

There were indications that NK activity might be involved in normal lymphopoiesis. Ross Basch (New York) presented preliminary evidence that the subpopulation of thymocytes killed by NK includes the large subcapsular cells which can 'home' back to the thymus in cell-transfer experiments. Rolf Kiessling then emphasized the remarkable similarity between NK cells and those that cause hybrid-histoincompatibility (in some F₁ hybrid combinations, there is a failure of haematopoietic stem cells from one of the parental strains to repopulate irradiated F₁ recipients). Not only were the H-2D linked genetic controls and inhibitors of NK activity and hybrid-histoincompatibility essentially the same, but up to 20% of normal bone marrow cells can be killed by NK, although no data were available as to which bone marrow cells were killed. Jonathan Sprent (Philadelphia University) noted that in similar F₁ hybrid combinations, mature parental T and B cells fail to recirculate or function. These results, taken together, suggest the NK activity may in normal *in vivo* conditions play some part in regulating lymphopoiesis.

Throughout the meeting, it was difficult to avoid the impression that NK and killer T-cells were in some way related. Since NK lysis can be a property of both the killer and the target, there is a resemblance between restricted T-cell killing and NK. Consider in the case of NK activity a cell-surface marker protein that is essentially constant from species to species and is subject to modifications due to viral infection, or even transformation, of the cell. If the NK system had a small repertoire of receptors capable of recognizing modifications of the marker protein, it might represent a surveillance system, albeit rather unspecific and inefficient. One therefore wonders if the NK system is a relic which recalls a stage in the evolution of contemporary killer T-cells. In an immunodeficient animal such as the athymic Nude, this relic could still provide valuable protection in the absence of a fully functional T-cell immune system. □

Natural Killer cells

from Rod Langman

NATURAL KILLER cells *in vitro* and *in vivo* was the theme of an Armand Hammer Cancer Workshop held at The Salk Institute, April 7-11, 1980. The three key questions that emerged from the discussions were, 1) Do NK cells show target specificity in killing? 2) Do NK cells operate as a tumour surveillance mechanism *in vivo*? 3) Have NK cells any other *in vivo* function?

NK activity can be specified by its lack of MHC restriction (which distinguishes it from T-cell killing) and the failure of anti-immunoglobulin reagents to inhibit it (which distinguishes it from ADCC or K-cell activity). Although it was thought that NK activity was tumour cell specific, there are now many exceptions to this generalization. It is now clear that there is some degree of specificity for particular cell lines or cell types, but it is not clear whether this is due to a difference in the target cells' ability to respond to NK lytic signals, or to a failure of the NK cells' ability to recognize the target, there being evidence for both possibilities.

The most convincing case of NK recognition specificity came from the cloned lines of NK cells described by Harvey Cantor (Sidney Faber Cancer Centre, Boston) which specifically kill target cells bearing a gp70 dependent determinant. Target cells not lysed by one NK clone could be rendered susceptible to lysis by infection with Moloney virus and killing by this NK clone could be inhibited by partially purified Moloney virus proteins.

NK clones, which carry the Thy 1 and Lyt 5 markers, were derived from normal spleen after removal of Lyt 1⁺ and Lyt 2⁺ cells, and cloned in 'T-cell growth factor' medium. There were reports by Ron Herberman (NIH, Bethesda), Rolf Kiessling (Karolinska Institute, Stockholm), Hans Wigzell (Uppsala), and Noel Warner (University of New Mexico, Albuquerque) all showing that NK cells from different sources gave unique patterns of NK lysis on panels of target cells.

The Lyt-5 marker has proved valuable in Cantor's hands for dissecting the regulatory activity of interferon on NK cells. A selected Lyt-5⁺, NK⁺ cell population upon incubation with interferon gains the Lyt-5⁺, NK⁺ phenotype, and at the same time itself begins to release interferon thus providing a positive feedback control loop. The rapid growth of interest in the anti-tumour effect of interferon means that this kind of control will attract increasing attention.

The question of specificity as a function of the ability of target cells to resist NK lytic signals was raised when John Collins (Salk Institute, La Jolla) reported that NK resistant fibroblast cell lines could be rendered NK sensitive by the addition of cycloheximide before or during the NK assay. There was some discussion whether cycloheximide inhibited a 'repair' process operating in target cells, or an interferon induced protection of NK lysis. While neither explanation was entirely satisfying, there is little doubt that target cells vary drastically in their sensitivity to NK lysis.

John Collins also reported that NK activity might be relevant to tumour surveillance *in vivo*. Starting with fibroblast

Rod Langman is Assistant Research Professor in the Developmental Biology Laboratory, The Salk Institute, La Jolla, California.

ARTICLES

Mid-Atlantic Ridge: zero-age geochemical variations between Azores and 22°N

H. Bougault*

Chief Scientist of the MAPCO Cruise, Centre Océanologique de Bretagne—BP 337, 29273 Brest, France

M. Treuil

Chief Scientist of the Project, Laboratoire de Géochimie Comparée et Systématique, 4, Place Jussieu, Tour 24/25 E3-75230 Paris, France

The MAPCO cruise of the RV J. Charcot sampled zero-age basalts along the Mid-Atlantic Ridge from the Azores Triple Junction to 24°N. Preliminary shipboard geochemical analyses together with earlier data show that some trace elements are not randomly distributed along the ridge. The latitude of the Hayes transform delineate two regions of rare earth element distribution: north from Hayes, is flat to light rare earth (or equivalent) enriched; south from Hayes, is light rare earth (or equivalent) depleted. These findings indicate large scale heterogeneity of the upper mantle.

THE IPOD program (International Phase for Ocean Drilling) has obtained many samples of igneous rocks from the ocean floor during the past five years. The studies related to this sampling and to samples collected by the more classical dredging method have provided many data which have been interpreted and discussed in terms of the fundamental processes of basalt genesis and the composition of the upper mantle. Some of these compositions are impossible to relate to one another by conventional magmatic processes of fractional crystallization, accumulation of crystalline phases and partial melting. In addition to these conventional processes, two main hypotheses have been proposed to account for the chemical heterogeneities in oceanic basalts that are observed at different scales. One of these hypotheses invokes magma mixing in one form or another; the second hypothesis assumes primitive heterogeneity of different source regions in the upper mantle. One interpretation does not preclude the other. Mixing, either of more or less fractionated liquids derived from the same source^{1–3} or of liquids derived from different sources^{4,5}, is necessarily restricted to local areas. The mantle heterogeneity concept can also be considered as a large-scale (regional) property^{6–9}. The research reported here is an attempt to examine regional heterogeneities along a portion of the Mid-Atlantic Ridge.

Area and techniques of analysis

The RV J. Charcot is equipped with a multi narrow-beam echo-sounding system (SEABEAM)¹⁰ which produces a real-time bathymetric map of the sea floor. The system scans an area whose width, normal to the ship's track, is two-thirds of the depth. Because it allows the immediate observation of large scale structures and of fine scale features (20-m contour

interval), the SEABEAM system permits relatively easy recognition of the rift valley and fracture zones (large-scale structures) and choice of a dredge site based on observation of fine scale features (such as, central high, volcano, steep slope). The objective of our analysis was to locate the inner floor of the central valley to dredge 'zero age' basalts. The rift valley area chosen for dredging was first surveyed by a track, approximately perpendicular to the ridge axis, roughly 30 miles long (15 miles to either side of the axis). This track was enhanced by a return profile located ~1.5–3 miles to the north or south of the initial crossing. In addition to providing information for dredging, each of these tracks constitutes data for structural interpretation. Dredge surveys and intermediate transits allowed us to obtain 54 transects of the ridge between 35°N and 24°N. Five profiles were made over each of three major transforms, Oceanographer, Hayes and Atlantis.

During this cruise, a new dredging system, 'active weight', was tried and used successfully. It is a percussion system: when the tension of the cable at the level of active weight, which is situated in front of the dredge, is approximately 3 tons, an impulse of 25 tons is communicated to the dredge. The system can act repeatedly at the bottom as it is reset by a return spring when no tension exists between the active weight and the dredge. Ten dredges along the ridge axis, with tracks of 0.5–1.5 miles, were attempted with this system and each dredge was half full to full of rocks. With this dredging method and with simultaneous use of SEABEAM, well positioned dredges can apparently be achieved with a minimum length of the dredge track, probably less than 0.5 miles.

Table 1 Different characteristics between the FAMOUS area (36°N) and sites 395, 396 (22°N). /Ch for Ta/La and Hf/La means normalized ratios*

	Ta/La ch	Hf/La ch	²⁰⁶ Pb/ ²⁰⁴ Pb	⁸⁷ Sr/ ⁸⁶ Sr	ΔNd
22°N (leg 45, 46)	0.56	2. – 2.6	18.0	0.7025	+13
FAMOUS	1.09	0.77 – 1.4	18.8	0.7028	+10

*Refs 20, 21.

*The members of the MAPCO (part 1 and 2) scientific team were: J. Y. Bervas, P. Beuzart, P. Cambon, M. El Azzouzi, G. Floch; J. Guichardot-Wirrmann, S. Monti, J. L. Olivet (Centre Océanologique de Bretagne), J. Durand, S. Savary (IPG St-Maur), C. Bassoulet, P. Tarits (IPG Paris), J. Argyriadis (Orsay), J. P. Eissen (Strasbourg), A. Y. Le Dain (Montpellier), A. Gourgaud (Clermont Ferrand), P. Fetter (Tunis), J. L. Joron, G. Meyer (Lab. P. Sue, CNRS), J. Stroup (Albany, US), A. Graham (British Museum (Natural History) London, UK) & D. Wood (Birmingham, UK).

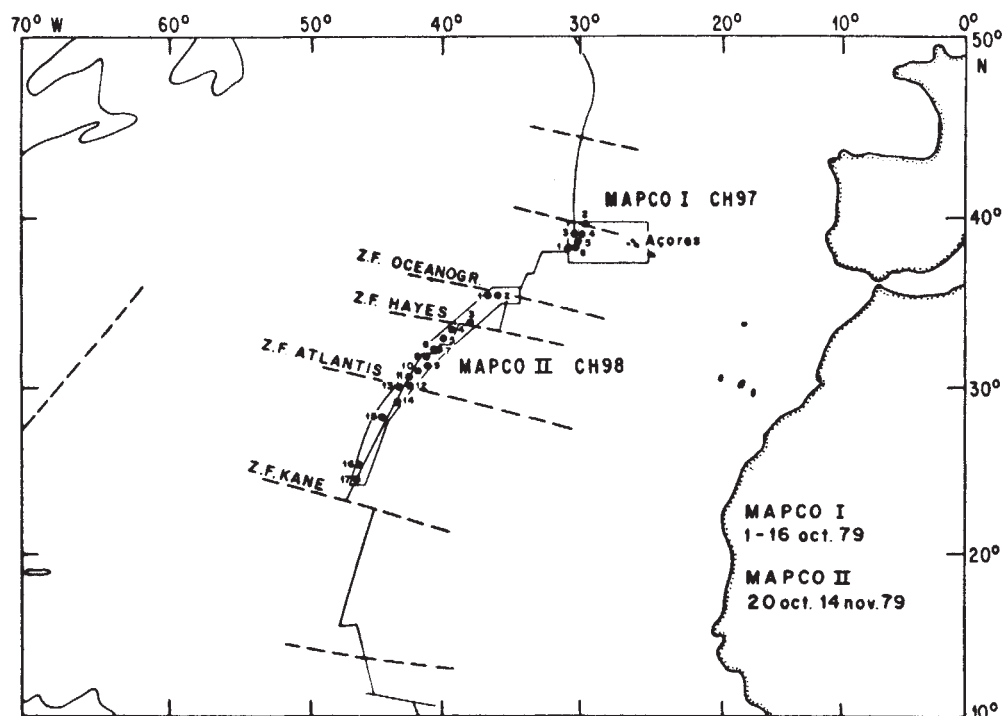


Fig. 1 Map of the studied area. Dots represent dredge sites.

Thin sections were made on board and an X-ray fluorescence unit, similar to the unit used on board the Glomar Challenger^{11,12}, allowed us to obtain selected trace element and major element data. The precision of the data is comparable to that of an onland laboratory: $\pm 1\%$ (relative) for major elements and ± 2 p.p.m. (absolute) for trace elements.

The primary objective of the first part of MAPCO (CH 97) was the structural analysis of the Azores Triple Junction between 39°N and 37°N (reported elsewhere); the primary objective of the second part of MAPCO (CH 98) and the secondary objective of MAPCO (CH 97) was to sample zero age basalts from the axis of the Mid-Atlantic Ridge. The secondary objective of MAPCO (CH 98) was to collect bottom topographic profiles, with emphasis on examining the progressive increase in average depth of the rift valley floor from North to South along the ridge.

Results and discussion

Six dredges were attempted proximal to the Azores Triple Junction during MAPCO (CH 97) and four of these were successful; 17 dredges were conducted as close as possible to zero age during MAPCO (CH 98) and 16 were successful (Fig. 1). The morphologic nature of basalt samples recovered during both parts of the cruise is varied. Two-thirds of the total recovered material is comprised of pillow basalt; one-quarter is comprised of segments of glassy crust varying from 1 to 10 cm in thickness and up to 40 cm in length. The remainder of the material is comprised of small broken fragments. Most of the samples are remarkably fresh and have clean vitreous surfaces. The pillow basalts exhibit characteristic radial fracturing, glassy margins, and have very well preserved extrusive forms of lava fingers or tubes up to 15 cm long. The basalt crusts or sheets are glassy on both sides; the presumed upper portion of these crusts has a ropy appearance and the glass is occasionally oxidized and veneered by sediment, while the lower surface contains portions of thin vertical walls of large cavities (up to 20 cm diameter) and/or frozen droplets of basalt. In dredges CH 98 11 and 12 we recovered massive glass blocks up to 15 cm diameter, glass shards, and thin flat sheets (1 cm thick) of glass. The crusts or sheets are interpreted to be either fragments of large pillows or tubes that were rapidly emptied, or fragments of surfaces of lava lakes¹³. In

addition to these basalt samples, scoriaceous basalts of probable subaerial origin were dredged at 900 m depth in the Azores Triple Junction area (CH 97 DR 05). One fragment of granitic metamorphic rock (garnet, cordierite) and a large number of blocks of sediment were recovered in dredge CH 98 DR 06 situated roughly 10 miles west of the rift valley axis.

Most of the basalt samples (two-thirds) are aphyric to subaphyric (phenocrysts $< 5\%$). Nearly all of these basalt fragments contain a few rounded plagioclase phenocrysts, possibly better referred to as xenocrysts, and one or two samples also contain rounded olivine phenocrysts. Microphenocrysts of euhedral olivine, quench olivine and plagioclase are all common. The remainder of the samples are porphyritic basalts (5–30% megacrysts, phenocrysts and/or microphenocrysts). Plagioclase is by far the dominant phenocryst phase; crystals are as large as 1 cm in length and are often rounded and partially reversely zoned. Some are better characterized as glomerocrysts. Most contain inclusions of glass or microcrystalline material. Olivine is a common, though usually minor, phenocryst phase. It is occasionally seen as rounded crystals up to 0.5 mm across but is generally subhedral to anhedral and fresh. Olivine contains rare small red-brown spinel. Clinopyroxene is observed infrequently as a phenocryst phase in these basalts, but is spectacularly present in dredge CH 97 DR 05 (in the Azores Triple Junction area) where it forms 15% of the rock.

Extensive geochemical data from the northern region (Azores Triple Junction and FAMOUS area) and the southern

Table 2 Duplicate analyses of three samples of CH 97 DR 02—Sr, measured from the same pellet preparations as for Nb, Zr and Y determinations, has been used to improve the classification of rock types within one dredge, because of its relative higher precision, Ti was measured from a glass disk preparation

	Sr	Nb	Zr	Ti	Y
101 a	138.0	14.1	75	7,020	26
101 b	136.4	13.6	78		
202 B a	141.6	13.1	76	6,900	26
202 B b	141.1	13.7	74		26
304 a	150.0	15.1	71	6,720	24
304 b	149.3	13.1	74		24

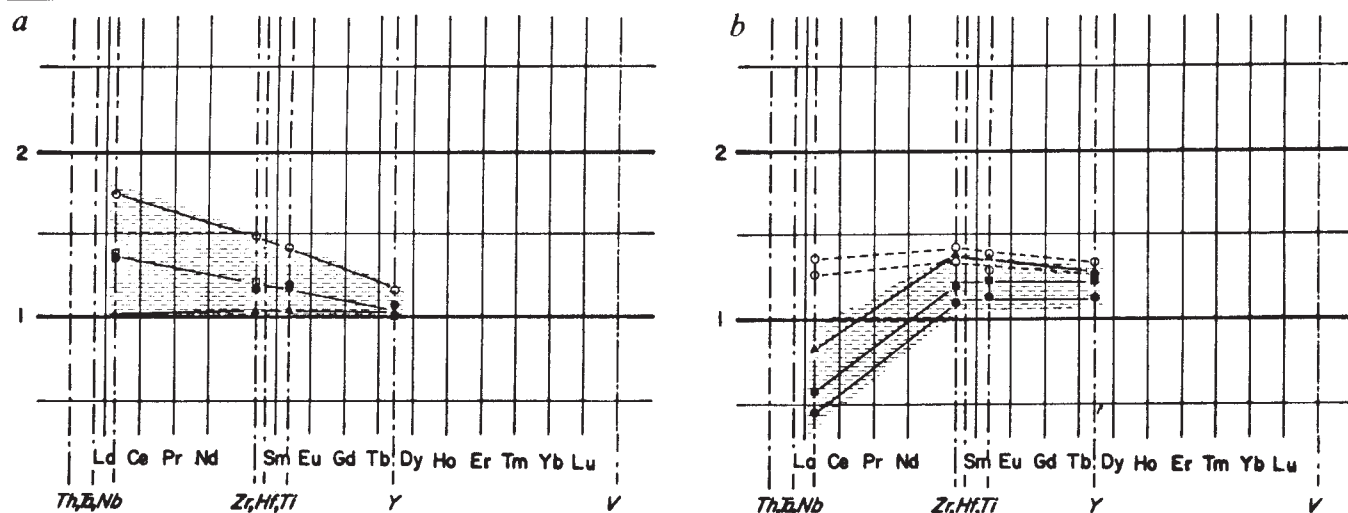


Fig. 2 Examples of extended rare earth diagrams including non rare earth hygromagmaphile elements. *a*, The spectrum observed in the Azores Triple Junction-FAMOUS area (data from refs 14, 16, 17, 23, 25); *b*, 22°N (data from refs 18, 19, 23). The preferred normalizing abundances corresponding to non rare earth elements are, in p.p.m.; Th, 0.028; Nb, 0.53; Ta, 0.031; Zr, 5.13; Hf, 0.128; Ti, 460; Y, 2.16; V, 22. In *a*, flat to light rare earth patterns, Nb, Ta and La plot at the same ordinate position (normalized Ta/La or Nb/La ratios are ≈ 1.09); *b*, light rare earth depleted patterns, Nb and Ta plot at a lower ordinate position than La (normalized Ta/La ratio is ≈ 0.5) see text.

region (22°N) have been interpreted as suggesting that there are significant differences in the isotopic compositions and trace element chemistry of the upper mantle sources between these two areas¹⁴⁻²⁰. Table 1 summarizes these data for the FAMOUS area and 22°N (ref. 21). Clearly from our present knowledge only isotopic data obtained from basalts can be interpreted unambiguously in terms of mantle sources. Hygromagmaphile element ratios such as Hf/La (similar to Zr/La or Sm/La) in basalts depend on both mantle source and partial melting. The two different ranges of Hf/La in the FAMOUS area and at 22°N are interpretable in terms of different mantle sources (one for each region) in agreement with the isotopic data. The range of this ratio in each area is probably due to different extents or ways of melting^{22,23}. As the main target of the MAPCO cruise is the examination of regional heterogeneities along a portion of the Mid-Atlantic

Ridge, namely the transition between the Azores Triple Junction-FAMOUS area and 22°N, selected hygromagmaphile elements or their ratios can help, at least in part, to describe these heterogeneities or this transition.

It has been shown for some samples that elements whose ions present a rare gas electronic structure can be included in the rare earth Coryell-Masuda plot^{24,25}; a comparative geochemical study of more than 300 oceanic basalts showing different rare earth patterns gave precise normalization values and relative behaviour of non rare earth hygromagmaphile elements compared with rare earth elements²⁶. Figure 2 presents such a diagram for samples from the Azores Triple Junction-FAMOUS region and from 22°N (refs 14, 19, 23, 25).

In the northern region the extended rare earth patterns are from flat to light rare earth enriched (Azores Triple Junction, FAMOUS, Sites 411, 412, 413 and 332, 334, 335 of DSDP);

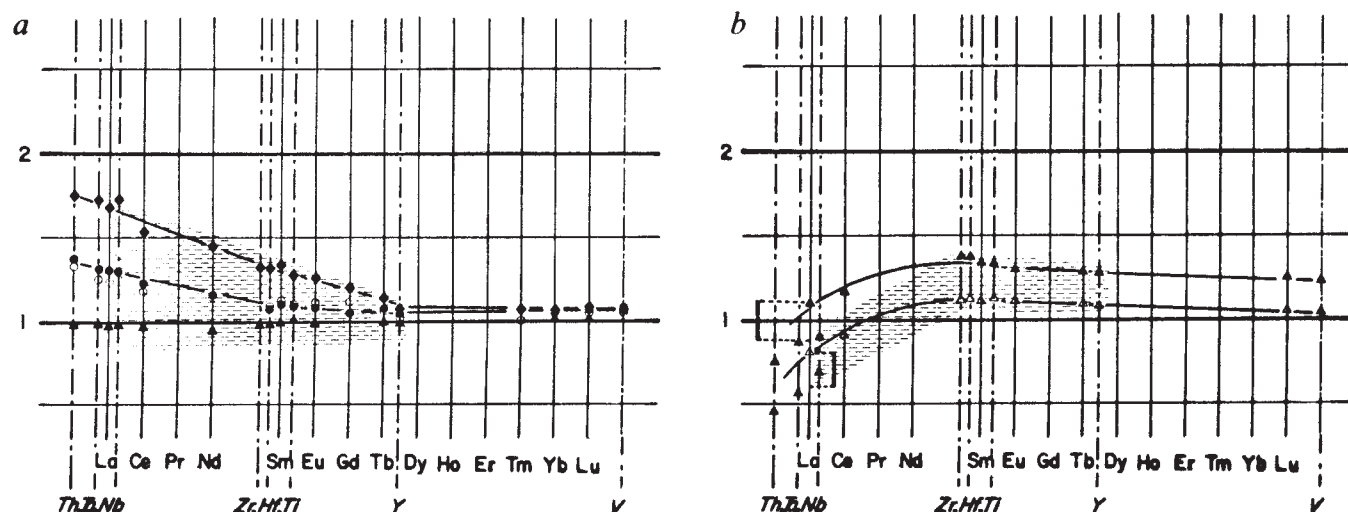


Fig. 3 *a*, Spectrum of non rare earth normalized patterns observed north from the Hayes transform. The end members of this spectrum are CH 97 DR 03 101, 39°N in the Triple Junction (○) and CH 98 DR 03 201, 33°N immediately north from Hayes fracture zone (FZ) (▲). The □, CH 97 DR 05 3... at 38°N, ● CH 98 DR 02 1... at 35°N (immediately south from Oceanographer FZ) have been chosen as examples showing similar distributions at different latitudes in this area. *b*, Spectrum of non rare earth normalized patterns observed south from Hayes transform. The plotted samples selected for examples are CH 98 DR 04, 33°N immediately south from Hayes FZ (●), CH 98 DR 07, 32°N (■) and CH 98 DR 17 301, 24°N (▲). The two exceptions (○) correspond to two groups in dredge CH 98 DR 08 at 31°N.

Table 3 Sr, Nb, Zr, Ti and Y data obtained on board; the figure in parentheses corresponds to the number of analysed samples

	Sr	Nb	Zr	Ti	Y
CH 97					
DR 02 See Table 1					
DR 03 39°06'N 29°54'W					
101 (1)	365	29	160	12,060	32
DR 05 38°42'N 29°57'W					
1.. (3)	397	28	155	11,460	27
2.. (4)	241	16	104	8,100	26
3.. (6)	192	13	86	6,840	21
DR 06 38°31'N 30°18'W					
1.. (6)	260	31	135	11,100	32
CH 98					
DR 02 35°11'N 36°14'W					
1.. (2)	155	12	75	7,400	26
201 (1)	110	8.5	74	8,220	33
DR 03 33°44'N 37°40'W					
1.. (2)	95	6	46	4,770	23
201 (1)	102	5	57	5,220	24
4.. (2)	92	6	61	5,850	25
DR 04 33°30'N 39°05'W					
1.. (4)	99	1.5	63	6,240	28
DR 05 33°02'N 39°20'W					
101 (1)	93	2	86	8,100	35
DR 06 32°17'N 40°21'W					
101 (1)	87	2	91	8,880	37
DR 07 32°17'N 40°11'W					
1.. (4)	93	2	80	8,000	34
DR 08 31°54'N 40°58'W					
1.. (3)	133	12	134	10,260	45
501 (1)	111	9.5	111	8,580	38
102 (1)	81	2	90	8,340	38
2.. (2)					
DR 09 31°28'N 40°57'W					
101, 301 (2)	91	2	87	8,460	36
201 (1)	89	1	69	6,780	30
DR 10 31°04'N 41°25'W					
101 (1)	110	3.5	113	10,080	42
2.. (2)	101	1.5	77	7,620	32
3.. (2)	75	1.5	71	6,960	31
DR 11 30°41'N 41°49'W					
1.., 3.., 4.. (7)	92	1.5	92	8,650	36
DR 12 30°10'N 41°55'W					
103, 204 (2)	92	2.5	103	9,600	41
DR 13 29°56'N 42°46'W					
204 et 301 (2)	122	3	105	8,940	36
DR 14 29°17'N 43°05'W					
101, 304 (2)	105	1.5	85	7,750	32
103, 201, 301 (3)	110	2.5	93	8,750	35
DR 15 27°46'N 44°05'W					
102, 301 (2)	113	1.5	95	8,190	32
2.. (2)	113	2.5	52	5,220	23
DR 16 25°16'N 45°20'W					
1.. (2)	128	3.0	100	8,300	33
201 (2)	119	3.5	112	9,360	39
202 (1)	124	5	92	7,320	30
DR 17 24°28'N 46°15'W					
101 (1)	148	3.8	121	9,300	35
201 (1)	167	2.6	99	7,260	31
301 (1)	136	3.5	125	10,260	38
401 (1)	138	3.0	110	9,000	34
Preferred chondrite abundance	0.53	5.13	460	2.16	

the normalized Ta/La or Nb/La ratio (≈ 1) is independent of the light rare earth enrichment (as well as for 45°N, site 410, and Reykjanes sites 407, 408 and 409). In the southern region (22°N) the extended rare earth patterns are systematically light rare earth depleted; the normalized Ta/La or Nb/La ratio is 0.5. Knowing this behaviour of hygromagmaphile elements, rare earths and non rare earths, it is possible to use Nb, Zr, Ti

and Y concentrations measured through X-ray fluorescence on board to obtain chondrite normalized diagrams equivalent to a rare earth diagram. The data may be interpreted in terms of mantle heterogeneity if these diagrams show sufficiently large differences as we know that a range of Sm/La or Zr/Nb ratios can be observed within a restricted area (FAMOUS and site 411, 412, 413 of DSDP).

Table 2 shows duplicate analyses of samples CH 97 DR 02 used to test the accuracy of measurements on board. Table 3 shows from north to south the results obtained during the cruise. Examination of these data indicates that the distribution of these elements is not random along the axis of the Mid-Atlantic Ridge as mentioned for rare earths by White and Schilling²⁷. Nb concentrations, the most hygromagmaphile element investigated, clearly show lower values in the southern region than in the northern region. The range of variation of (Nb, Zr, Ti, Y) chondrite normalized patterns are shown in Fig. 3. North from the Hayes transform these patterns are flat to Nb enriched (equivalent to light rare earth enriched); south from the Hayes transform they are Nb depleted (equivalent to light rare earth depleted); one exception in the southern region is observed in CH 98 DR 08 where two groups of samples show flat or only slightly Nb depleted patterns.

The large difference of the chondrite normalized plots of Nb, Zr, Ti and Y (equivalent to rare earth plots) between north and south from Hayes transform allow us to interpret these preliminary results in terms of regional mantle heterogeneity. The suggested limit (latitude of the Hayes transform) will have to be confirmed by isotopic data. It will be also interesting to study the Ta/La ratio between the Hayes transform and 22°N to confirm whether or not the single normalized Ta/La ratio found so far (≈ 0.5) for light rare earth depleted material comparatively to the single value (≈ 1) observed for flat to light rare earth enriched materials.

The MAPCO cruise was supported by the Groupe Croute Océanique du Comité IPOD—France, the CNRS and CNEXO. We thank J. Bonnin for help in formulating this program and D. Needham for assistance with the cruise plan. The active weight system used during the cruise was developed by J. P. Allenou with the support of J. Y. Bervas. We also thank the Captain, the officers and the crew of the RV J. Charcot. Contribution no. 685 from the Département de Géophysique, Géologie et Géochimie marines du C.O.B.

Received 16 January; accepted 25 April 1980.

- O'Hara, M. J. *Nature* **266**, 503–507 (1977).
- Rhodes, J. M., Dungan, M. A., Blanchard, D. P. & Long, P. E. *Tectonophysics* **55**, 35–61 (1979).
- Bryan, W. B., Thompson, G. & Michael, P. G. *Tectonophysics* **55**, 63–85 (1979).
- Schilling, J. G. *Nature* **242**, 565–571 (1973).
- Sun, S. S., Tatsumoto, M. & Schilling, J. G. *Science* **190**, 143–147 (1975).
- O'Nions, R. K., Hamilton, P. J. & Evensen, N. M. *Earth planet. Sci. Lett.* **34**, 13–22 (1977).
- Tarney, J., Wood, D. A., Varet, J., Saunders, A. D. & Cann, J. R. *Deep Drilling Results in the Atlantic Ocean: Oceanic Crust, Maurice Ewing Ser. 2*, 285–301 (1979).
- Bougault, H., Joron, J. L., Treuil, M. *Deep Drilling Results in the Atlantic Ocean: Oceanic Crust, Maurice Ewing Ser. 2*, 352–368 (1979).
- Proc. R. Soc.* (in the press).
- Renard, V. & Allenou, J. P. *Int. Hydrogr. Rev.* **56**, 35–67 (1979).
- Bougault, H. *Init. Rep. DSDP Leg 37*, 643–657 (1977).
- Melson, W. G., Rabinowitz, P. D., Natland, J. H., Bougault, H. & Johnson, H. P. *Init. Rep. DSDP Leg 45*, 5–20 (1979).
- Francheteau, J., Juteau, T. & Rangin, C. *Nature* **281**, 209–211 (1979).
- O'Nions, R. K. & Pankhurst, R. J. *Init. Rep. DSDP Leg 37*, 599–602 (1977).
- Drach, V., Müller-Sohnns, D., Köhler, H. & Huckenholz, H. G. *Init. Rep. DSDP Leg 45*, 535–590 (1977).
- Puchelt, H., Emmermann, R. & Srivastava, R. K. *Init. Rep. DSDP Leg 37*, 581–590 (1977).
- Schilling, J. G., Kingsley, R. & Bergeron, M. *Init. Rep. DSDP Leg 37*, 591–598 (1977).
- Rhodes, J. M., Blanchard, D. P., Dungan, M. A., Rodgers, K. V. & Brannon, J. C. *Init. Rep. DSDP Leg 45*, 447–460 (1979).
- Bougault, H., Treuil, M. & Joron, J. L. *Init. Rep. DSDP Leg 45*, 493–506 (1979).
- Bougault, H., Joron, J. L. & Treuil, M. *Proc. R. Soc.* (in the press).
- Allègre, C. J., Brévart, O., Dupré, B. & Minster, J. F. *Proc. R. Soc.* (in the press).
- Langmuir, C. H., Bender, J. F., Bence, A. E., Hanson, G. N. & Taylor, S. R. *Earth planet. Sci. Lett.* **36**, 133–156 (1977).
- Bougault, H., Cambon, P., Corre, O., Joron, J. L. & Treuil, M. *Tectonophysics* **55**, 11–34 (1979).
- Sun, S. S., Nesbitt, R. W. & Sharaskin, A. Ya. *Earth planet. Sci. Lett.* **44**, 119–138 (1979).
- Wood, D. A. *et al. Earth planet. Sci. Lett.* **42**, 77–97 (1979).
- Bougault, H., Joron, J. L. & Treuil, M. (in preparation).
- White, W. H. & Schilling, J. G. *Geochim. cosmochim. Acta* **42**, 1501–1516 (1978).

Flow patterns in the central North American ice sheet

W. W. Shilts

Terrain Sciences Division, Geological Survey of Canada, 601 Booth Street, Ottawa, Canada K1A 0E8

Patterns of glacial dispersal of lithologically distinctive erratics around Hudson Bay show the central portion of the North American Laurentide ice sheet to have been made up of at least two land-based centres, one that grew and dissipated in Keewatin, and one that grew and dissipated in Nouveau Quebec-Labrador.

AN accurate model of the flow patterns of the central portion of the North American ice sheet is of fundamental importance in reconstructing the palaeogeography of northern North America for the past million or more years. The configurations of the margins of the Laurentide sector of the Wisconsinan ice sheet during the last 10,000 yr of its existence have been detailed satisfactorily, but inferences about the ice flow patterns during glacial maxima and locations and growth mechanisms of centres of glacial outflow have been hampered by a paucity of data from the Canadian Arctic and sub-Arctic. Most inferences on these earlier events have been drawn indirectly from geophysical data, from limited isostatic data on raised and tilted lacustrine or marine shorelines, and from broad, large-scale reconnaissance mapping of glacial features (largely by airphoto interpretation).

Over the past decade much relatively detailed information has been obtained on the lithological character of glacial sediments, the orientation of ice-inscribed or ice-moulded features, and on the general stratigraphic framework of glacial sediments around Hudson Bay. These data, by defining paths of glacial transport of distinctive bedrock lithologies, permit the postulation of several constraints on timing and configuration of glacial flow for the central parts of the Laurentide ice sheet (at least as it was constituted during the Wisconsinan). These constraints have led to a preliminary model for glacial flow that conflicts with many aspects of recently published ice sheet reconstructions¹⁻³. Furthermore, although the model is crude, its gross features are unequivocal and are sufficiently accurate to use as a basis for speculating on the configurations, palaeoclimatological conditions and dynamics of the last Laurentide ice sheet.

The data on which the maps and conclusions of this article are based are principally the petrological compositions of till and related sediments from: (1) the Hudson Bay Lowlands and adjacent areas of the Canadian Shield; (2) southern District of Keewatin; and (3) Coats and Mansel Islands (Fig. 1). The petrology of tills has been characterized by determining the percentages of several distinctive pebble lithologies in the 2-6-mm fractions of ~1,200 samples of surface and older tills. Pebble lithologies and geochemical and mineralogical dispersal patterns were noted for a further 7,000+ samples of till collected in southern District of Keewatin⁴⁻⁶. Field observations of erratic types present at each of several hundred localities throughout these regions have been integrated with the quantitative data, as have pebble lithologies of glacial or related bottom sediments collected from Hudson Bay by C. F. M. Lewis and B. V. Sanford.

The petrological data are supported by observations of orientations of ice-inscribed or ice-moulded features compiled or generalized from the Glacial Map of Canada⁷, from measurements of striation directions on boulders or boulder pavements in tills exposed in stratigraphic sections in the

Hudson Bay Lowlands, and from measurements of orientation of striations on bedrock, of fluting, and of minor moraine or drumlins within a 200,000-km² area where detailed mapping of surficial geology has been carried out in southern Keewatin and on Coats and Mansel Islands.

Bedrock sources of erratics around Hudson Bay

Figure 1 shows the locations of the major geological domains within and around Hudson Bay. The older Precambrian terrane is largely underlain by non-calcareous, crystalline gneiss and igneous intrusive rocks. These 'crystalline' rocks include major belts of deformed, metamorphosed volcanogenic and associated sedimentary strata that generally retain some of their original lithological characteristics.

Superimposed on the 'crystalline' terrane are belts of Proterozoic sedimentary and volcanogenic rocks that are generally much less affected by metamorphism and which contain lithologically distinctive facies. One of these, the Dubawnt Group⁸, comprises a sequence of generally red sedimentary and volcanogenic formations that underlie an area of several tens of thousands of square kilometres west and south-west of Baker Lake.

Another Proterozoic depositional basin, the Circum-Ungava geosyncline⁹, forms an almost continuous belt of sediments, including locally significant occurrences of iron formation, basic volcanic rocks, and basic or ultrabasic igneous rocks. The sediments of that part of the basin in and adjacent to Hudson Bay are predominantly dark grey to black greywackes, slates, various facies of iron formation, and minor dolomitic units.

A thick sequence of gently dipping, unmetamorphosed Palaeozoic carbonate rocks with minor clastic facies underlies much of Hudson Bay and extends for several hundred kilometres on land south and south-west of the Bay¹⁰.

Because of the distinctive lithological differences among the erratics derived from each of these major geological domains, pebble lithologies in the tills around Hudson Bay are grouped into four general and easily defined categories. For convenience, these four types of erratics will be referred to as crystalline erratics, red erratics, dark erratics, and carbonate erratics, in order of the above discussion of their source areas.

Outcrop patterns of these four lithological types are such that by mapping the areal distribution of the glacial erratics derived from each, one can place constraints on the glacial flow paths that must have prevailed during transport of the erratics from their source outcrops. In this way flow lines can be generalized for the central part of the Laurentide ice sheet. By establishing patterns of dispersal of erratics for the surface drift, and by comparing lithologies of older glacial deposits with the youngest, inferences bearing on the similarity or dissimilarity of flow patterns for older glacial events may be

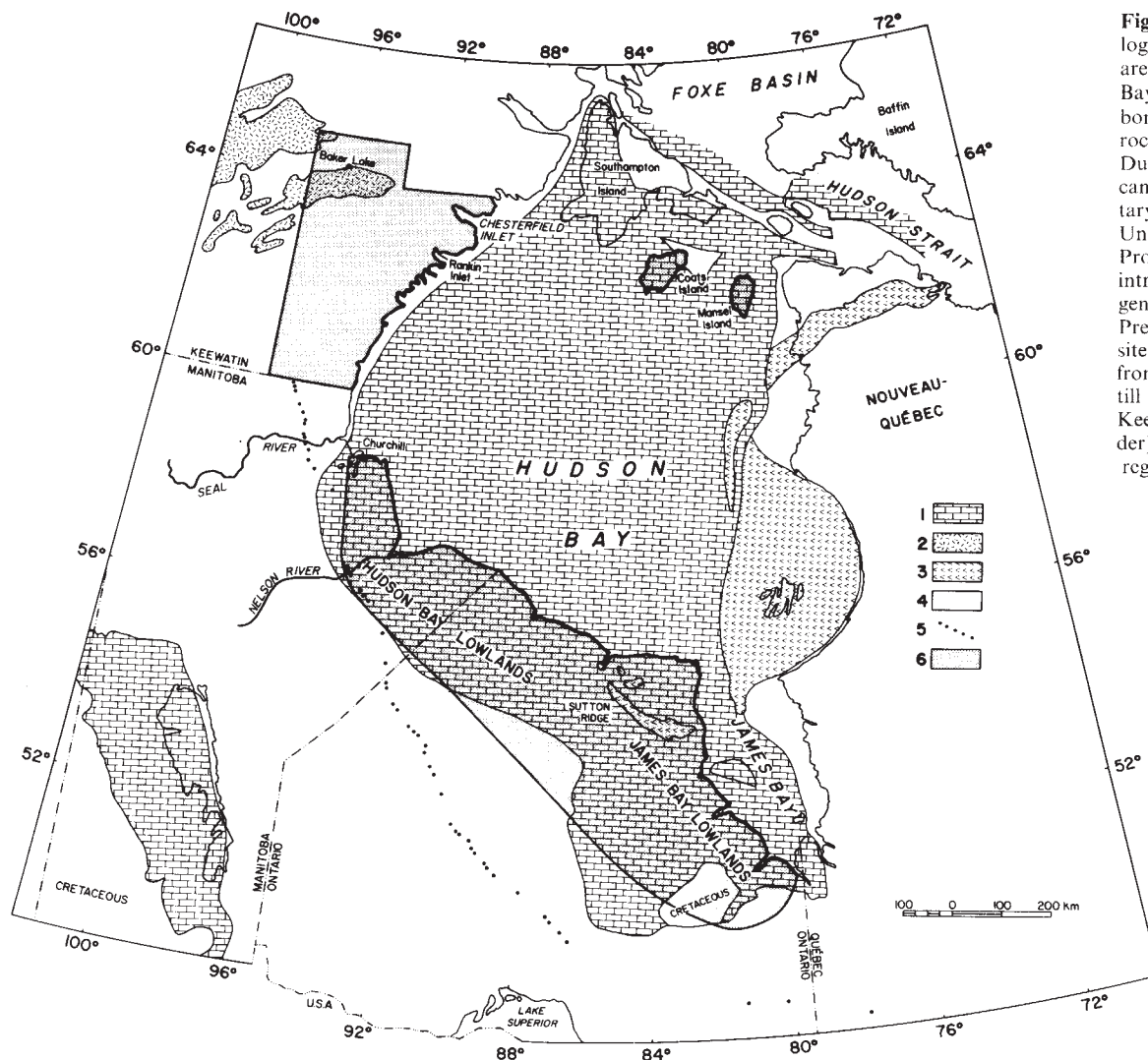


Fig. 1 Generalized geology and principal study areas around Hudson Bay. 1, Palaeozoic carbonate and minor clastic rocks; 2, Proterozoic Dubawnt Group, red volcanogenic and sedimentary rocks; 3, Circum-Ungava geosyncline, Proterozoic sedimentary, intrusive, and volcanogenic rocks; 4 crystalline Precambrian terrane; 5, sites of samples collected from boreholes through till (not shown north of Keewatin-Manitoba border); 6, principal study regions discussed in text.

evaluated. Furthermore, by documenting the distance travelled by erratics during any one glacial event, one can make rough calculations of the time required for the distance of transport, as has already been done for the Dubawnt (red) erratics mapped on the mainland west of Hudson Bay¹¹.

Dispersal patterns around Hudson Bay

Dispersal of Carbonate erratics: south and south-west of Hudson Bay, carbonate erratics have been displaced hundreds of kilometres westward and southward onto crystalline Precambrian bedrock (Fig. 2). Carbonate erratics (20–30%) have been found in the fine gravel fraction of fine-grained, calcareous till 250 km from the nearest carbonate outcrops. The northern limit of westward carbonate dispersal is sharply defined in the vicinity of Seal River, just north of Churchill, Manitoba (L. A. Dredge, personal communication). Till from boreholes drilled north of Seal River bears no Palaeozoic carbonate erratics and has a non-calcareous matrix at least as far north as Chesterfield Inlet. South of James Bay, the Ontario-Quebec border (long. 79°30') roughly divides the area of carbonate dispersal to the west from the area of non-calcareous drift to the east. Other than in the area of the late glacial Cochrane readvance, south-east of James Bay, no evidence has been found of eastward dispersal of Palaeozoic erratics east of James Bay or Hudson Bay (refs 12–14, and J. S. Vincent, personal communication). The rare Palaeozoic and Proterozoic erratics found east of the Bay and cited by Lee¹⁵

as evidence of eastward dispersal are confined to zones of postglacial marine submergence and were undoubtedly emplaced by some form of ice rafting¹³.

Although calcareous Palaeozoic debris could have been displaced eastward or westward from ice centred on Hudson Bay at some earlier time and could have been eroded and transported back into or across the Bay during later flow from Quebec or Keewatin centres, one would expect that at least some of the calcareous debris would have been left behind to be incorporated into the drift that now covers the areas adjacent to Hudson Bay. Certainly in other areas, where later ice flow has been at considerable angles to earlier flow, some components of the earlier flow have been left behind, although their frequencies are attenuated and their dispersal patterns modified. Such relict evidence of earlier dispersal has been observed in southern Keewatin¹¹ and in the Appalachian region of southern Quebec¹⁶ where later major ice movements have not completely effaced earlier dispersal patterns.

Dispersal of dark erratics: dark grey to black sedimentary and volcanic rock fragments are widely distributed in drift throughout much the same area as that covered by till rich in carbonate erratics (Fig. 2). Isolated observations of dark erratics, iron formation and jasper have been interpreted^{17,18} to indicate a source within the Circum-Ungava Proterozoic sequence that both underlies the eastern part of Hudson Bay and projects through the Palaeozoic cover in the Sutton Ridge¹⁰. V. K. Prest (personal communication, 1979) has noted widespread occurrence of dark erratics and oölitic jasper erratics in northwestern Ontario at least 1,000 km west of their

nearest possible Proterozoic sources in association with fossiliferous Palaeozoic carbonate erratics over 600 km west of their nearest source. Dark erratics and associated iron formation commonly comprise 10–20% of the erratics (2–6 mm) in tills lying on Palaeozoic outcrops west and south-west of Hudson and James Bays. West of Hudson Bay, dark erratics are rare (generally $\leq 5\%$) north of Seal River, and those that are found could reasonably be related to local sources within greenstone belts. Dark erratics make up at least 50% of the Precambrian erratics on Mansel Island and are found there in association with haematite-magnetite iron formation and other erratics which are common lithologies within the Circum-Ungava geosyncline⁹. Dark erratics are found on the east coast of Coats Island but are uncommon west of there.

Dark erratics are also found in older tills in numerous exposures throughout the Hudson Bay Lowlands and in a series of 6–20-m deep boreholes drilled in multiple tills overlying crystalline terrane west of the Lowlands. At given sites within the Lowlands, the concentrations of dark erratics (and also crystalline erratics) are generally similar among till sheets, including till sheets which predate the last interglacial stage^{19,20}.

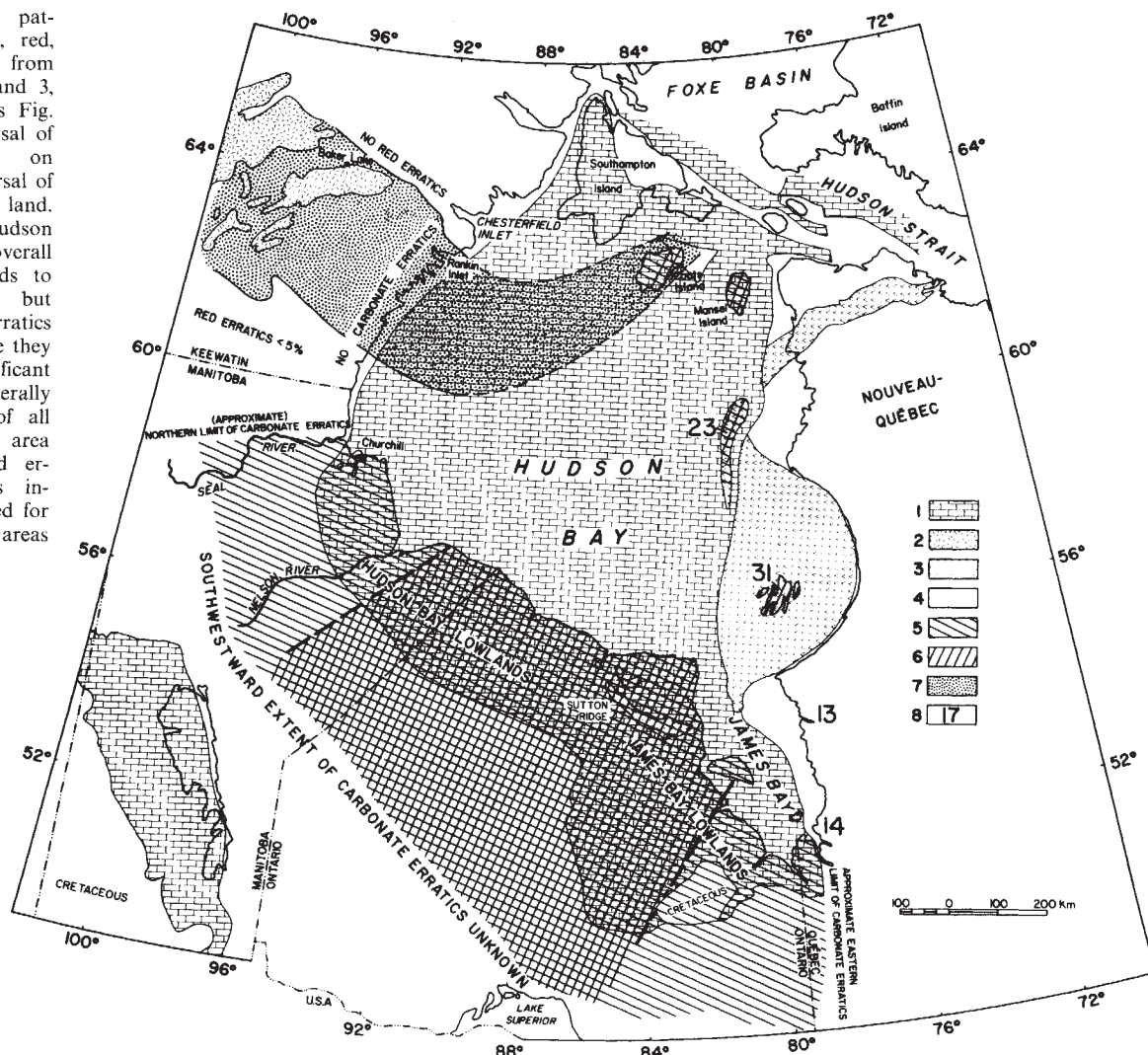
Dispersal of red erratics: the pattern of dispersal of red erratics has been discussed in detail elsewhere¹¹ for that portion of the district of Keewatin east of the Keewatin ice divide. No red erratics occur north of a south-east-trending line from Baker Lake to the coast of Hudson Bay, just north of Rankin Inlet. In this region a well-developed southeastward-trending dispersal train of red erratics is

superimposed on a poorly defined southerly train created during an earlier glacial flow event. Red erratics comprise 1–2% of the fine gravel in eskers and till on Coats Island. Distinctive cobble and boulder-size fragments of the red Pitz and Christopher Island Formations of the Dubawnt Group are numerous on Coats Island and can be ascribed confidently to outcrops that occur exclusively within a 100 km long belt that extends southwestward from the east end of Baker Lake (A. N. Lecheminant, personal communication).

No red erratics were found on Mansel Island, nor were they found in till-like bottom sediments from the floor of Hudson Bay between Coats and Southampton Islands. Red erratics comprise 1% of the gravel fractions of till samples near the Manitoba border, but no certain red erratics have been found as far south as Churchill. Although the true pattern of dispersal of red erratics between the Keewatin mainland and Coats Island is poorly known, the main part of the dispersal train seems to narrow northwards and to make at least a 90° bend, a configuration confirmed by the pattern of distribution of red erratics in till-like sediments from the bottom of Hudson Bay.

Dispersal of crystalline erratics: crystalline erratics are found virtually everywhere that it has been possible to make observations in the region covered by Figs 1 and 2. Crystalline erratics make up ~10% of the gravel fractions of all tills sampled in the southern Palaeozoic portion of the Hudson Bay Lowlands and make up 20% or more of the gravel-sized erratics occurring in tills from Hayes River–Nelson River northward. Crystalline erratics make up the bulk of the Precambrian erratics on both Coats and Mansel Islands. In the

Fig. 2 Dispersal patterns of carbonate, red, and dark erratics from source areas 1, 2 and 3, respectively. 1–4, as Fig. 1; 5, area of dispersal of carbonate erratics on land; 6, main dispersal of dark erratics on land. South-west of Hudson and James Bays overall dispersal corresponds to carbonate pattern but dispersal of dark erratics is only shown where they make up a significant proportion, generally more than 15% of all erratics; 7, principal area of dispersal of red erratics; 8, numbers indicate references used for data on erratics in areas indicated.



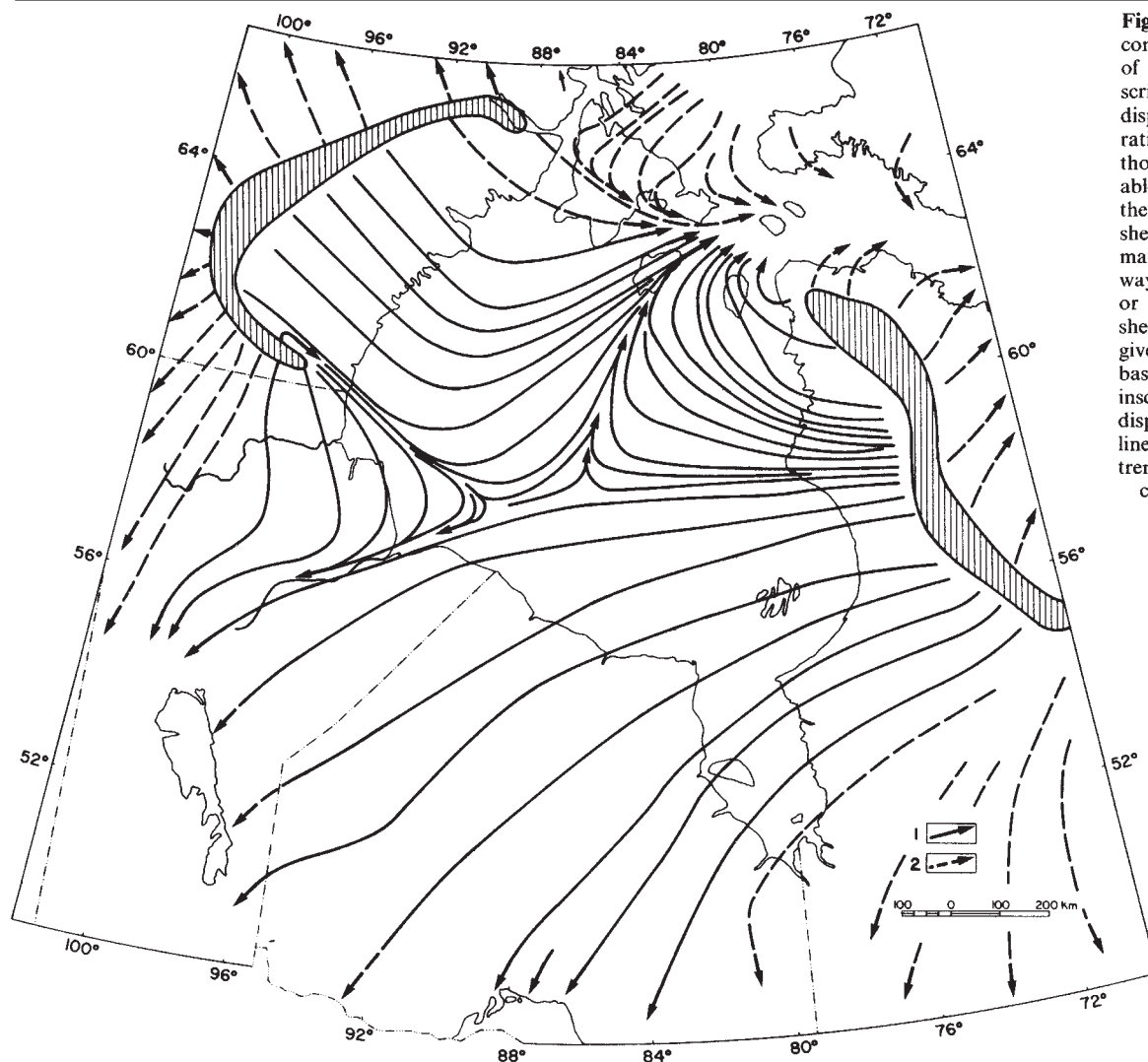


Fig. 3 Ice flow lines reconstructed from trends of ice moulded or inscribed features and from dispersal patterns of erratics. Although this is thought to be a reasonable reconstruction for the central part of the ice sheet at the last glacial maximum, there is no way to tell precisely how or where the two ice sheets interfaced at any given time. 1, Flow lines based on ice moulded or inscribed features and on dispersal patterns; 2 flow lines based on ice flow trends shown on the glacial map of Canada⁷.

regions underlain by crystalline bedrock, crystalline erratics generally make up more than 95% of the gravel fraction, except down-ice from major dispersal areas of more erodible, less metamorphosed outcrops, such as the Palaeozoic carbonates or Dubawnt red beds.

In the district of Keewatin, geochemical and mineralogical dispersal patterns from several mineralized or mineralogically distinctive bedrock sources within the crystalline terrane east of the Keewatin ice divide invariably indicate eastward or southeastward glacial transport towards Hudson Bay.

Directions of flow from striation measurements

Numerous observations have been made (refs 19, 20 and B. G. Craig, unpublished data) of southwesterly oriented striations on boulder pavements within or between till sheets and on bedrock throughout the southwestern part of the James Bay and Hudson Bay Lowlands. Skinner's data clearly show that all tills preserved south-west of James Bay, including pre-Missinaibi (pre-Sangamon) tills, were deposited by glacier ice flowing from east-northeast. An exception to this general flow pattern is an area of clay-rich till associated with southeasterly oriented drumlins and striations that were probably formed during the Cochrane readvance²¹, a southward and eastward late glacial surge. Observations^{19, 20, 22} of southeasterly oriented striations on till stones and on Precambrian bedrock near the western edge of the Hudson Bay Lowlands from Churchill to the Ontario-Quebec border indicate that although southwesterly flow dominated all glaciations of this

region, at some time during at least one glaciation, ice flow was southerly to southeasterly.

Hardy¹⁴, among others, has shown that striations preserved in the eastern part of the Hudson Bay Lowlands invariably indicate ice flow westward or southwestward toward Hudson Bay. Andrews and Falconer²³ show that ice flow across the Ottawa Islands in the east-central part of Hudson Bay varied from northeastward to westward.

On Coats and Mansel Islands several directions of striae and fluting were noted, most being northward or northeastward, sub-parallel to the long axes of those islands. On Coats Island, however, the last direction of ice flow was eastward, presumably directly from Southampton Island or northern Keewatin.

In areas outside those discussed above striation directions are known or assumed to be similar to the main trends shown on the glacial map of Canada⁷.

Discussion

Traditionally, the concepts regarding the configuration of the central part of the Laurentide ice sheet have been derived largely from observations of the amount of isostatic uplift of marine and lacustrine shorelines and from gravity data that seem to indicate that a significant amount of isostatic adjustment has yet to be made over Hudson Bay. These data have led to the belief that the locus of greatest ice loading, and hence the centre of glacial outflow at the maximum development of the last Laurentide ice sheet, was over Hudson Bay itself, notwithstanding the abundant glacial

geomorphological evidence from regions adjacent to the Bay, all of which indicates ice flow radially away from centres of dispersal located in Keewatin and Nouveau Quebec. The terrestrial centres or ice divides have been explained as late glacial phenomena²⁴, formed when marine waters split a Hudson Bay ice dome into eastern and western segments with concomitant readjustment of ice flow patterns. Thus, the geomorphological features were thought to have been formed during the last 2,000–4,000 yr of the ice sheet's existence.

However, the time-transgressive nature of marine limit¹¹ combined with significant amounts of restrained rebound^{11,25} in areas of marine inundation make interpretation of maximum uplift data difficult around Hudson Bay. In addition, recent measurements of marine limit on southeastern Southampton Island (124–157 m)²⁶, Coats Island (124 m) and the Ottawa Islands (156–163 m)²³ are lower than would be expected if the northern or north-central part of Hudson Bay had been loaded more heavily than adjacent areas on land. Vincent and Hardy²⁷ have shown convincingly that lacustrine strandlines which predate the marine incursion south-east of James Bay, are tilted upward towards a centre of uplift in Nouveau Quebec and not towards a centre over Hudson Bay as would be expected if an ice dome had existed there.

Although there is no firm evidence relating to the origin of the gravity anomalies that are found in parts of Hudson Bay, crustal blocks within the Bay seem to be in equilibrium with their adjacent terrestrial counterparts²⁸, suggesting that the anomalies may be in part a permanent feature of the crust under the basin.

From the above dispersal and geomorphological data, a map (Fig. 3) has been prepared showing probable flow directions in the central part of Laurentide ice sheet near a glacial maximum, but at an unspecified point in time.

The dispersal patterns of Fig. 2 show that an ice dome or domes could not have existed in the Hudson Bay basin unless some special conditions existed, such as the presence of a melt lake³ or a frozen bed under the central portion of the ice sheet. Such conditions would have prevented erosion and transportation of the Palaeozoic carbonate rocks from beneath the Bay. Two facts that argue against special conditions are the consistent eastward dispersal patterns from distinctive sources in the crystalline terrain east of the Keewatin ice divide, well above and west of the central depression, and the abundant evidence of repeated episodes of intense erosion of the Palaeozoic and Proterozoic rocks that underlie the southern half of the Hudson Bay depression. The configuration of erratic dispersal patterns suggests, rather, that independent ice masses existed on either side of Hudson Bay.

The well defined northern limit of westwardly displaced carbonate lithologies along the Seal River in northern Manitoba indicates that ice from the Quebec centre never affected the western shore of Hudson Bay north of that river. The presence of red erratics on Coats Island and their absence on Mansel Island indicates that ice from a Keewatin centre was confined to the western portions of Hudson Bay, but the presence of dark erratics on Coats Island suggests that at some time ice from a Quebec centre flowed at least as far west as Coats Island. In any case, the dispersal patterns at the mouth of the Bay indicate that terrestrial ice flow centres were established well before marine waters divided the ice sheet. The lack of carbonate erratics east of Hudson Bay supports the interpretation that the pattern of westward dispersal of carbonate and dark erratics west and south-west of Hudson Bay was not related to a centre of dispersal within the Bay but to a centre on land to the east.

The general picture conveyed by the reconstruction depicted in Fig. 3 is one of independent Quebec and Keewatin ice masses in contact along discrete zones. The zone of contact in the northern part of the Bay was more or less between Coats and Mansel Islands. The contact zone depicted through the Hudson Bay Lowlands could have been as far north as Seal River and as far south as approximately the Quebec–Ontario

border, based on the limits of carbonate dispersal. It is drawn at the Nelson River in this reconstruction because the amount of crystalline erratics in youngest tills north of Nelson River is considerably higher than in tills south of the river. The moderate concentrations of crystalline erratics in tills south of the river are assumed to have their origin principally in the crystalline terrain east of Hudson Bay. North of Nelson River the frequency of crystalline erratics doubles, and because there is no reason that a source area east of Hudson Bay would cause this enhancement, it is thought that much of the crystalline component was carried southward by Keewatin ice, from sources north-west of the lower reaches of Nelson River.

The shift through time of the contact of the Quebec and Keewatin ice masses in the southwestern Hudson Bay Lowlands explains the commonly observed presence of two sets of striae, one indicating flow from north or north-west, and one indicating flow from east or north-east. When the glacier from a Quebec–Labrador centre flowed over a given site, the latter sets of striae were formed; when Keewatin ice displaced the Quebec ice at the same site, the former directions prevailed. The strong evidence of westward to southwestward dispersal through the western Hudson Bay Lowlands suggests, however, that the Keewatin centre had a minor role in most of the Lowlands, either by virtue of its inability to erode and transport or because it did not flow long enough over most of the region to effect significant erosion and transportation.

That the position through the Hudson Bay Lowlands of this zone of contact between Quebec and Keewatin ice masses seems to have shifted considerable distances with time probably reflects the relative strengths and timing of ice build-up in the two centres of ice dispersal. It is tempting to speculate that within the central part of the Laurentide ice sheet the Labrador–Quebec centre of outflow predominated in the early and maximum stages of glaciation and that the Keewatin centre became relatively stronger during the later stages. Certainly in the last stages of glaciation there was a significant break in the vicinity of the Quebec–Ontario border between ice retreating towards a centre in Quebec and ice retreating generally northward or northwestward towards a Keewatin centre. The Cochrane readvance^{14,30} seems to have been independent of the Quebec ice and may, therefore, have emanated from an ice mass shrinking toward a Keewatin centre.

The magnitude of the distances of transport of all erratics, more than 1,000 km in the case of the carbonate and dark erratics and at least 600 km in the case of the red erratics, demonstrates that ice flow was maintained from Keewatin and Quebec centres of outflow along discrete flow paths for significant periods of time, as already suggested elsewhere^{11,30}. The duration of ice flow necessary to effect these distances of transport for erratics of any given till sheet must have been of the order of several tens of thousands of years if the reasoning used by Shilts *et al.*¹¹ or by Boulton *et al.*²⁹ is followed.

That erratic transport occurred along specific flow lines over such long periods of time seems to be incompatible with the concept that there could have been any prolonged period of glacial flow with a configuration other than that depicted on Fig. 3. Thus, it is concluded from the constraints of time and configuration of dispersal discussed above that ice flow was maintained from centres located east and west of Hudson Bay throughout one or more glacial stages of the Wisconsin Stage. Lithological similarities between pre-Wisconsinan and Wisconsinan tills in the Hudson Bay Lowlands suggest that similar centres also existed during earlier glaciations.

Conclusions

In the region around Hudson Bay, the configuration of dispersal patterns for distinctive suites of glacial erratics indicates that a centre of ice flow or ice dome could not have existed over the Bay unless some special conditions existed that prevented erosion and transportation of debris from the Bay's limestone floor. Dispersal data agree with glacial

geomorphological data in that they indicate ice flow from centres on land in Keewatin and in Nouveau Quebec-Labrador.

The distance of dispersal along clearly defined flow paths from the centres east and west of Hudson Bay is so great that the time required to form the major erratic trains of the Wisconsinan glaciation may have spanned most of that glacial stage, leaving little time for the development of a major ice dome over Hudson Bay.

Thus, I conclude that it is unlikely that an ice dome could have existed over Hudson Bay during the Wisconsinan nor is it likely, based on compositions and striated boulder pavements of pre-Wisconsinan tills of the Hudson Bay Lowlands, that an ice dome existed during the earlier glacial stages represented by these deposits. Centres of ice dispersal are thought, rather, to have developed and decayed on land east and west of Hudson Bay.

Received 26 November 1979; accepted 22 April 1980.

- White, W. A. *Geol. Soc. Am.* **83**, 1037 (1972).
- Hughes, T., Denton, G. H. & Grosswald, M. G. *Nature* **266**, 596 (1977).
- Sugden, D. E. *J. Glaciol.* **21**, 367 (1978).
- Shilts, W. W. *Boreas* **6**, 203 (1977).
- Klassen, R. A. & Shilts, W. W. *Inst. Min. Metall. Prosp. Areas of Glac. Ter.* **80** (1977).
- DiLabio, R. N. W. & Shilts, W. W. *Geol. Surv. Can. Pap.* 77-1A, 479 (1977).
- Prest, V. K., Grant, D. R. & Rampton, V. N. *Geol. Surv. Can. Map* 1253A (1970).
- Donaldson, J. A. *Geol. Surv. Can. Pap.* 64-20, 1 (1965).
- Dimroth, E., Baragar, W. R. A., Bergeron, R. & Jackson, G. D. *Geol. Surv. Can. Pap.* 70-40, 45 (1970).
- Sanford, B. V., Grant, A. C., Wade, J. A. & Barss, M. S. *Geol. Surv. Can. Map* 1401A (1979).
- Shilts, W. W., Cunningham, C. M. & Kaszycki, C. A. *Geology* **7**, 537 (1979).
- Hillaire-Marcel, C. *Cah. Géogr. Québ.* **20**, 185 (1976).
- Dionne, J.-C. *Rev. Geogr. Montréal* **28**, 453 (1974).
- Hardy, L. thesis, McGill Univ. (1976).

Hudson Bay received ice flowing from Keewatin and Quebec throughout the last glacial stage. Reconstructions of the palaeoclimatology and dynamics of the Laurentide ice sheet might better be based on the multiple dome model presented here rather than on the less complex 'single dome' model.

Samples and unpublished data on which the discussions of glacial flow southwest of Hudson Bay are based were provided by Polar Gas Consortium through W. D. Roggensack and by B. C. McDonald, R. G. Skinner, B. G. Craig and C. F. M. Lewis. Canadian Coast Guard icebreaker CCGS Pierre Radisson provided helicopter support and a logistical base for study of Coats and Mansel Islands. Analyses of samples from Keewatin were partially funded by Atomic Energy of Canada Limited. Most of the analyses of pebble fractions were carried out by C. A. Kaszycki and P. Honarvar.

- Lee, H. A. *Geol. Soc. Am.* **70**, 219 (1959).
- McDonald, B. C. & Shilts, W. W. *Geol. Soc. Am.* **76**, 683 (1971).
- Bell, R. *Geol. Surv. Can. A. Rep.* 1886 pt G, 1 (1887).
- Prest, V. K. *Geol. Surv. Can. Pap.* 63-66, 1 (1963).
- McDonald, B. C. *Geol. Surv. Can. Pap.* 68-53, 78 (1969).
- Skinner, R. G. *Bull. geol. Surv. Can.* **225**, 1 (1973).
- Hughes, O. I. *Geol. Soc. Am. Spec. Pap.* 84, 535 (1965).
- Tyrrell, J. B. *Ont. Bur. Mines* **22** pt 1, 161 (1913).
- Andrews, J. T. & Falconer, G. *Can. J. Earth Sci.* **6**, 1263 (1969).
- Lee, H. A., Craig, B. G. & Fyles, J. G. *Geol. Soc. Am.* **68**, 1760 (1957).
- Andrews, J. T. *Geol. Surv. Can. Pap.* 68-53, 49 (1969).
- Bird, J. B. *Acta geogr. Univ. Iudz.* **24**, 75 (1970).
- Vincent, J.-S. & Hardy, L. *Géogr. Phys. Quat.* **31**, 357 (1977).
- Hall, D. H. *Geol. Surv. Can. Pap.* 68-53, 337 (1969).
- Boulton, G. S., Jones, A. S., Clayton, K. M. & Kenning, M. J. *British Quaternary Studies, Recent Advances* 231 (Clarendon, Oxford, 1977).
- Prest, V. K. *Geol. Surv. Can. Econ. Rep.* **1**, 676 (1970).
- Jackson, G. D. *Geol. Surv. Can. Pap.* 60-20, 1 (1960).

Invertible DNA determines host specificity of bacteriophage Mu

Pieter van de Putte, Steve Cramer & Micheline Giphart-Gassler

Department of Molecular Genetics, State University of Leiden, Wassenaarseweg 64, 2333 Al Leiden, The Netherlands

The function of the invertible G region of bacteriophage Mu is apparently to confer different host specificities on Mu. Two products of genes S and U, situated in the G region are not needed for the infectivity of Mu G(−) particles. In the Mu G(−) phage the S gene product and the 21-K polypeptide, presumably the product of gene U, are missing. Instead, two other polypeptides with different molecular weights are observed.

THE invertible G region of bacteriophage Mu is 3,000 base pairs long and has short inverted repeats at its ends¹. When Mu is propagated lytically in *Escherichia coli* K12 the G region is fixed in one particular orientation G(+), whereas when Mu stocks are prepared by induction of a Mu *cts* prophage the DNA consists of 50% G(+) and of 50% G(−) molecules^{1,2}.

A Mu gene *gin* situated in the β part of the Mu genome (Fig. 1) is essential for the inversion of the G area³. Only G(+) particles can infect *E. coli* K12 (refs 3, 4), but it has been speculated that the G(−) particles may be infectious for organisms other than *E. coli* K12 (ref. 5).

We now provide evidence that Mu G(−) particles are viable and have a different host specificity from G(+) particles.

Propagation in *Citrobacter freundii*

It has been reported that Mu can be propagated in cells of *C. freundii* but that the organism seemed to have, besides a restriction system directed against foreign DNA in general, a second restriction system specifically directed against phage

Mu, because Mu plated with a low efficiency on *C. freundii* r[−] strains⁶. However, the presence of such a specific restriction system seems unlikely. Another explanation is that *C. freundii* cells are not sensitive to the Mu G(+) phages, which are the predominant product of lytic growth on *E. coli* K12, but to the G(−) form. To test this, the efficiencies of plating (e.o.p.) of different Mu phage preparations were determined on *E. coli* K12 and *Citrobacter* hosts. It seems that the lytically propagated Mu phage Muc2000, a clear plaque mutant of Mu c⁺, has an extremely low e.o.p. of 2.5×10^{-9} on the r[−] strain of *Citrobacter* (Table 1, line 1) whereas the e.o.p. of a Muc⁺ lysate obtained by induction is the same on *E. coli* K12 as on *C. freundii* (Table 1, line 2). This simple experiment strongly suggests that *C. freundii* is sensitive for Mu G(−) phages. (The nine log difference found between the e.o.p. of lytic Mu and induced Mu on *C. freundii* is much larger than could be expected from the e.o.p. data obtained by De Graaf *et al.*⁶. This difference is a result of the way the Mu phage was prepared by these authors: a spontaneous induction lysate of a Muc⁺ lysogen with $\sim 10^5$ plaque-forming units (PFU) per ml^{−1} consisting of 50% G(+) and 50% G(−) particles was

directly used to make confluent lysis plates on *E. coli* K12. The lysate of $\sim 10^9$ PFU ml $^{-1}$ obtained in this way still contained 10^5 G(-) particles which led to an e.o.p. of nearly 10^{-4} on the *C. freundii* r $^{-}$ strain.) That the orientation of the G area is involved in this phenomenon follows also from the behaviour of induced Mucts gin^3 phages. In this Mu mutant G is fixed in the + orientation and consequently the phages form no plaques on the *Citrobacter* host (Table 1, line 3).

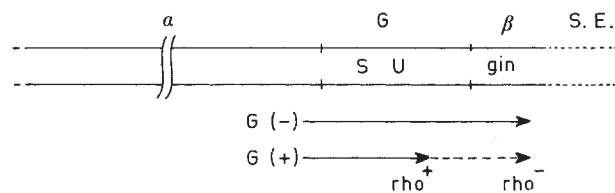


Fig. 1 A model for the expression of *gin* during lytic development of Mu G(+) and Mu G(-) phages.

G inversion during lytic development

When Muc2000 was propagated lytically on *C. freundii* and titrated on different hosts it was found that the e.o.p. on an *E. coli* K12 r $^{-}$ host was much higher (factor of 10^6) than when lytically propagated Mu on *E. coli* K12 was plated on *Citrobacter* (compare lines 1 and 5, Table 1). Apparently, G inversion is more frequent during lytic development of G(-) phages than of G(+) phages. A likely explanation for this is that the expression of *gin* is dependent on the orientation of the G area. In the + orientation the G area might act as a transcription barrier for *gin* expression, if the *gin* promoter is located in the α region of Mu (Fig. 1). This idea is supported by the following result: when Muc2000 is propagated on an *E. coli* K12 *suA* strain, which is defective in the transcription termination factor ρ (ref. 7), the relative number of G(-) phages in a G(+) lysate is much enhanced (Table 1, line 4). In the prophage state expression of *gin* leads to a randomization of the orientation of the G region. However, our experiments show that Mu phages propagated lytically on *E. coli* K12 normally contain very few G(-) phages. This suggests that in the prophage state *gin* is expressed from a different promoter and that this promoter is shut off during lytic development.

Propagation on other hosts

Other *E. coli* strains and bacterial species were tested for sensitivity to Mu G(+) and G(-) phages. With induced Mucts62 phage we found plaques on lawns of *E. coli* C and of *Shigella sonnei*. Two *Klebsiella* strains, *Salmonella typhimurium*, an *Aerobacter* strain, and *E. coli* B were found to be Mu resistant.

Muc2000 G(-) phages propagated on *C. freundii* form plaques on lawns of *E. coli* C and *S. sonnei* with an efficiency approximately a factor 10^7 higher in comparison with Muc2000 G(+) propagated on *E. coli* K12 (Table 1, lines 1 and 5). This suggests that *S. sonnei* and *E. coli* C are also sensitive to Mu G(-) phages.

However, there are some complications. As *E. coli* C is deficient in restriction functions the e.o.p. of induced Mucts on *E. coli* C should be 1. In fact the e.o.p. on *E. coli* C and on *S. sonnei* is much lower than 1 and, moreover, is difficult to determine accurately. Besides the few normal plaques found, a large number of extremely faint plaques were seen. This is probably due to a poor adsorption of Mucts62 to *E. coli* C and *S. sonnei* which is not related to G inversion. Phage Muc2000, which compared to Mucts62 adsorbs better to *E. coli* K12, also forms uniform clear plaques on *E. coli* C and *S. sonnei*. However, when these phages are propagated on *E. coli* C or *S. sonnei* and plated on *Citrobacter* or vice versa (Table 1, lines 5 and 7) still mutual differences in the e.o.p. of a factor 10–30 are observed. The reason for these differences is not yet known and was not further investigated.

Restriction enzyme analysis

The orientation of the G region is easily determined using restriction enzymes such as *HpaI*, which cut asymmetrically in the G region⁸. Figure 2 shows the analysis of different DNAs treated with *HpaI*. The *HpaI* digest of Mu DNA derived from phage lysates prepared on *E. coli* K12 is shown in lane b. The *HpaI*-E fragment of about 3,500 base pairs contains part of the G region. Mucts62 DNA obtained from an induced phage lysate contains an additional *HpaI* fragment of $\sim 3,800$ base pairs derived from the 50% G(-) molecules (Fig. 2, lane c). Of these two *HpaI* fragments only the 3,800 base pair fragment is present in the DNA of Mu phage propagated in *C. freundii* (lane d). The G region must be almost exclusively in the G(-) orientation in this DNA because the fragment present in the Mu G(+) has disappeared. Mu DNA from phage propagated on *C. freundii*, *E. coli* C and *S. sonnei* (lanes d–f) gave the same restriction pattern.

Genes in the G region

Amber mutations in the genes *S* and *U* have been mapped inside the G region⁹. The G(-) phage cannot infect cells of *E. coli* K12 which might indicate that the *S* and *U* gene products are not made during lytic development of G(-) phages. A likely explanation for this is that *S* and *U* genes are expressed from a promoter located outside the G region in the α part of Mu (see Fig. 1). The expression of these genes from such a promoter will then be blocked after G inversion. To test whether the *S* and *U* gene products are essential for Mu G(-) phages we made induced lysates from an *E. coli* K12 strain lysogenic for MuctsSam and another which is lysogenic for MuctsUam and determined the titres on different strains lacking nonsense suppressors. Normal titres were found on the

Table 1 Plating efficiencies of Mu phage lysates on different hosts

Phage	Mode of propagation	Host	Efficiencies of plating				
			<i>E. coli</i> K12 r $^{-}$	<i>E. coli</i> K12 r $^{+}$	<i>C. freundii</i> r $^{-}$	<i>S. sonnei</i> r $^{+}$	<i>E. coli</i> C (r $^{-}$)
1 Muc2000	lytic	<i>E. coli</i> K12	1	1	2.5×10^{-9}	$< 10^{-9}$	$< 10^{-9}$
2 Mucts62	induced	<i>E. coli</i> K12	1	1	1	(10^{-6})	(4.10^{-5})
3 Mucts62 gin	induced	<i>E. coli</i> K12	1	1	$< 10^{-9}$	$< 10^{-9}$	$< 10^{-9}$
4 Muc2000	lytic	<i>E. coli</i> K12 $supA$	1	1	10^{-6}	—	—
5 Muc2000	lytic	<i>C. freundii</i>	2.6×10^{-3}	2.0×10^{-6}	1	8.5×10^{-3}	7.5×10^{-2}
6 Muc2000	lytic	<i>S. sonnei</i>	3.1×10^{-4}	—	6.9×10^{-2}	1	2.5
7 Muc2000	lytic	<i>E. coli</i> C	1.0×10^{-5}	—	2.7×10^{-2}	2.0×10^{-1}	1

All lytically propagated phage were made by the confluent lysis method. When the phage is propagated on a particular strain and titrated on the same strain, the efficiency of plating (e.o.p.) is arbitrarily set at 1. The e.o.p. values for induced Mucts62 on *S. sonnei* and *E. coli* C (line 2) are given in parentheses as these values were extremely variable and dependent on plating conditions. All titrations were on 1.8% agar plates containing 1% Difco trypton broth; 0.8% NaCl and 0.5% Difco yeast extract.

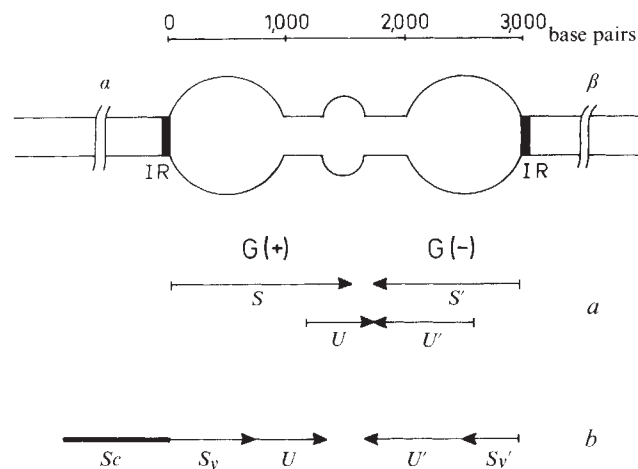


Fig. 4 Two models for the organization of the G region of bacteriophage Mu. In model *a*, gene *S* is completely inside the G region based on the observation that no *S* ambers were found to map outside the G region⁹. The position of gene *U* is drawn as far as possible to the right taking into account that the rightmost 1,300 base pairs of the G region are non-essential for Mu G(+) phages³. Gene *U* could also be located completely inside gene *S*. The presence of separate genes *S'* and *U'* for Mu G(-) phages is supposed because the *S* and *U* gene products are non-essential for Mu G(-) particles. The sizes are based on the appearance of two new polypeptides in Mu G(-) virions instead of the *S* and *U* gene products which have disappeared (Fig. 3). The drawing of the heteroduplex structure of G is according to the EM measurements of Chow *et al.*¹³. In model *b*, the *S* and *U* genes do not overlap and there is a common part of *S* and *S'* indicated with *Sc* outside the G region. The genes *S* and *S'* differ from each other in the part located inside G, indicated with *Sv* and *Sv'*, respectively.

barrier for the expression of a gene *gin*. The *gin* gene is situated outside the invertible region in the β part of Mu and is needed for the inversion of the G area³. It is also of interest that the gene *mom* is located adjacent to *gin* (A. Toussaint, personal communication) and might be coordinately expressed with *gin*. The *mom* gene codes for a modification function of a general nature¹². If the function of G inversion is to prepare the phage for entering new hosts it will be extremely advantageous for the phage if a modification function is expressed simultaneously with *gin* to protect the phage DNA against foreign restriction enzymes.

Organization of the invertible region

All amber mutations in the genes *S* and *U* have been mapped inside the invertible region G. *S* codes for a 56,000-MW protein while *U* probably codes for a 21,000-MW polypeptide¹⁰. We have shown that these two gene products are only essential for Mu G(+) phages as *S* and *U* amber mutants plate on *C. freundii*, which is sensitive to Mu G(-) phages. Therefore, we postulate that two other genes which we indicate as *S'* and *U'* are also situated in the G region and are expressed in Mu G(-) phages instead of the *S* and *U* genes. We have some experimental evidence for the existence of *S'* and *U'*. The *S* and *U* gene products are present in Mu virions propagated in *E. coli* K12 which is sensitive to Mu G(+). However, these proteins are absent in Mu G(-) virions and are replaced by two new polypeptides with molecular weights of 48,000 (presumably *S'*) and 26,000 (presumably *U'*). More recently, we have isolated amber mutants of Mu G(-) phages which are likely candidates for mutants in the genes *S'* and *U'*. Three of these amber mutants seem to behave as non-ambers in *E. coli* K12. They fall in two complementation groups and are rescued by λ -pMu hybrid phages containing the β and G regions of Mu.

As some 4,100 base pairs are needed to code for *S*, *S'*, *U* and *U'* their genes have to partially overlap if they are to be

accommodated in the G region. Probably *S* and *U* overlap each other as only 1,700 base pairs of DNA are available to code for *S* and *U*. This follows from the fact that 1,300 base pairs of the right part of G can be deleted in Mu G(+) phages without loss of viability³. We propose that *S'* and *U'* genes are situated in the 1,300 base pairs that can be deleted in the Mu G(+) phages. Such a model is presented in Fig. 4*a*. However, if *S* is situated partially outside the G region, it is not necessary to postulate overlapping genes (see Fig. 4*b*). The presence of G-like structures in P₁ and P₇ (refs 13, 14) supports model *a*. It is tempting to suggest that G is a unit involved in adsorption which has or had in the past the capacity to transpose itself to other DNA molecules. In fact the G region shows similarities with transposable elements as this region is bordered by inverted repeats and a *recA* independent recombination system (*gin*) acts on these repeats. If model *b* is correct then a striking analogy exists with immunoglobulins. The polypeptide chains in an immunoglobulin molecule have a constant region and a variable region coded for by separate genes which are fused, probably by a deletion, a transposition or an inversion^{15, 16}. According to Fig. 4*b*, *S* and *S'* would also consist of a constant common part coded for by a region outside the G area and a variable part located inside G. A more precise determination of the position of *S* and analysis of the four proteins will reveal whether model *a* or *b* is correct.

It is important to consider how a structure like the G region was created: it contains two pairs of genes, one pair being silent while the other is expressed. From electron microscopic studies it seems that the middle part of the G region, about 1,000 base pairs, shows an inverted homology except for a small part around the centre (see also Fig. 4). This suggests a rather extensive homology between *S* and *U* on one hand and *S'* and *U'* on the other hand. Such an extensive homology within G could be explained if G has evolved from a tandem duplication of *S* and *U* followed by an inversion of one duplicate. Subsequently one duplicate could have changed in such a way that adsorption to other hosts became possible. The nature of these changes is not yet known but apparently involves at least the sizes of the structural tail fibre proteins (*S'* < *S* and *U'* > *U*). It is of interest that we have found mutants of Mu that make slightly larger or smaller *S* proteins. These were found during the screening in minicells of amber mutants of Mu unrelated to *S*, indicating that changes in *S* might occur with a rather high frequency. In this way Mu might maintain a high degree of flexibility to adapt to changes in the environment in respect to the availability of host receptor sites.

Analogies with G inversion

Several other organisms exhibit regulatory mechanisms analogous to G inversion in Mu in that gene expression is controlled by genetic rearrangement. One example is the phase variation observed in *Salmonella typhimurium*¹⁷. This organism produces two types of flagella encoded by two different genes. The orientation of a promoter situated on an invertible region of 800 base pairs adjacent to one of the structural genes determines which of the two genes is expressed. Another analogy is with the regulation of the different mating type elements in yeast, α and α , which are expressed only after transposition from a silent position in the chromosome to the mating locus (cassette model¹⁸). In this type of mechanism, structural genes (genetic 'cassettes') are moved and thus activated. G inversion in Mu has aspects of both types of mechanism: like phase variation, host range switching in Mu involves inversion; like mating type inversion, the structural genes themselves move.

We thank Dr Jan van der Embden whose observations on G-like structures in *S. aureus* phages stimulated the initiation of our experiments, and Dr Ira Herskowitz for interesting discussions on cassette-type controls. This work was supported by Euratom grant 194-76 Bian to M.G.G.

Received 21 December 1979; accepted 16 April 1980.

1. Daniell, E., Abelson, J., Kim, J. S. & Davidson, N. *Virology* **51**, 237 (1973).
2. Hsu, M. T. & Davidson, N. *Proc. natn. Acad. Sci. U.S.A.* **69**, 2823 (1972).
3. Kamp, D., Kahmann, R., Zipser, D., Broker, T. R. & Chow, L. T. *Nature* **271**, 577 (1978).
4. Symonds, N. & Coello, A. *Nature* **271**, 573 (1978).
5. Howe, M. M. *Nature* **271**, 608 (1978).
6. De Graaf, J., Kreuning, P. C. & Van de Putte, P. *Molec. gen. Genet.* **123**, 283 (1973).
7. Richardson, J. P., Grimley, C. & Lowery, C. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1725 (1975).
8. Allet, B. *et al.* in *DNA Insertion Elements, Plasmids and Episomes* (eds Bukhari, A. I. *et al.*) 745 (Cold Spring Harbor Laboratories, New York, 1977).
9. Howe, M. M., Schumm, J. W. & Taylor, A. L. *Virology* **92**, 108 (1979).
10. Giphart-Gassler, M., Wijffelman, C. A. & Reeve, J. N. *J. molec. Biol.* (submitted).

11. Magazin, M., Reeve, J. N., Maynard-Smith, S. & Symonds, N. *FEMS Microbiol. Lett.* **4**, 5 (1978).
12. Toussaint, A. *Virology* **70**, 17 (1976).
13. Chow, L. T. and Bukhari, A. I. *Virology* **74**, 242 (1976).
14. Toussaint, A. *et al.* *Virology* **89**, 146 (1978).
15. Tonegawa, S., Hozumi, N., Matthiessen, G. & Sculler, R. *Cold Spring Harb. Symp. quant. Biol.* **41**, 877 (1976).
16. Honjo, T. & Kataoka, T. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2140 (1978).
17. Zeig, J., Hilmen, M. & Simon, M. *Cell* **15**, 237 (1978).
18. Hicks, J. B., Strathern, J. N. & Herskowitz, I. in *DNA Insertion Elements, Plasmids and Episomes* (eds Bukhari, A. I. *et al.*) 457 (Cold Spring Harbor Laboratories, New York, 1977).
19. Laemmli, U. K. *Nature* **227**, 680 (1970).

A mouse α -globin-related pseudogene lacking intervening sequences

Elio F. Vanin, Gregory I. Goldberg, Philip W. Tucker* & Oliver Smithies

Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706

A mouse α -globin-related pseudogene ($\psi\alpha 30.5$) completely lacks intervening sequences, and could not code for a functional globin polypeptide because of frameshifts. The widespread occurrence of globin pseudogenes in other species suggests that they are not 'dead' genes but may be important in controlling globin expression.

INTERVENING SEQUENCES (introns) interrupt many eukaryotic genes¹⁻⁵. In productive globin genes, that is, those producing functional globin polypeptides, these sequences are transcribed but are removed from the primary nuclear transcripts before the resulting mRNA is translated in the cytoplasm (reviewed in refs 6, 7). The functions of intervening sequences are not known.

The mouse genome has at least seven non-allelic globin genes: two adult β genes (β^{maj} and β^{min}), two β -type embryonic genes (γ and ζ), two adult α genes and an α -type embryonic gene (x). An adult α and two adult β genes have been cloned and sequenced⁸⁻¹³. Each of the two β -globin genes has at the same position in the coding region a small 116-base pair intervening sequence^{11,13} which has only three nucleotide differences between the two genes¹³. A second larger intervening sequence, 650 base pairs long in β^{maj} and 628 base pairs in β^{min} , differs considerably in sequence between these two genes¹³, although it is in the same position in both. Intervening sequences have been found in the coding regions of all productive β -type globin genes described to date, including the adult β -type genes from rabbit^{4,14}, chicken¹⁵ and man¹⁶, and the human fetal β -type globin genes, G_γ and A_γ (ref. 17). An adult α -globin gene⁹ from BALB/c mouse has also been shown to contain two intervening sequences, 122 and 134 base pairs long, which occur in positions analogous to those of the β -type globins. A human adult α -globin gene has comparable intervening sequences (ref. 18, and S. Liehaber and Y. W. Kan, personal communication).

We describe here the nucleotide sequence of an α -globin 'pseudogene', which we call $\psi\alpha 30.5$, previously isolated^{19,20} from the DNA of outbred CD1 Swiss mice (Charles River Mouse Farms). $\psi\alpha 30.5$ is clearly related to the productive mouse α -globin gene, but it lacks the two intervening sequences found in the productive gene; the intervening sequences are as cleanly missing as in fully processed mRNA. This unusual gene also has several additions and deletions, from 1 to 12 base pairs long, relative to the sequenced productive mouse α -globin gene⁹. These additions and deletions introduce frameshifts which would prevent $\psi\alpha 30.5$ from producing a functional α -globin polypeptide; this is why we refer to it as an α -globin pseudogene.

Nishioka, Leder and Leder (personal communication) have recently isolated and sequenced the gene equivalent to $\psi\alpha 30.5$

from BALB/c mice, and we have found that AKR/J and SWR/J mice have an *EcoRI* fragment of DNA in their genomes comparable in size and hybridization properties to the $\psi\alpha 30.5$ fragment. Globin pseudogenes are also found in other species^{18,21-23}, and this widespread occurrence raises many questions about their evolution and function.

Restriction map of $\psi\alpha 30.5$ and sequencing strategy

The α -globin pseudogene 30.5 was isolated, as clone Charon3AMm30.5 (refs 19,20), from a random collection of recombinant bacteriophages containing *EcoRI* fragments of liver DNA from the Swiss mice. Figure 1a shows the restriction enzyme map of the 4.7-kilobase pair *EcoRI*

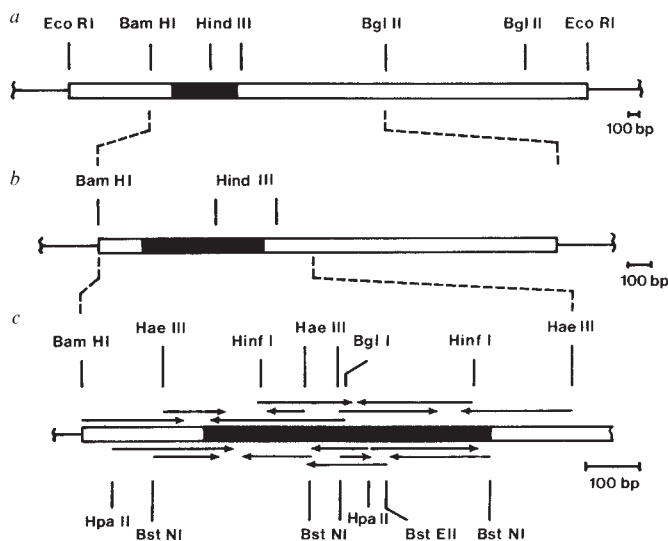
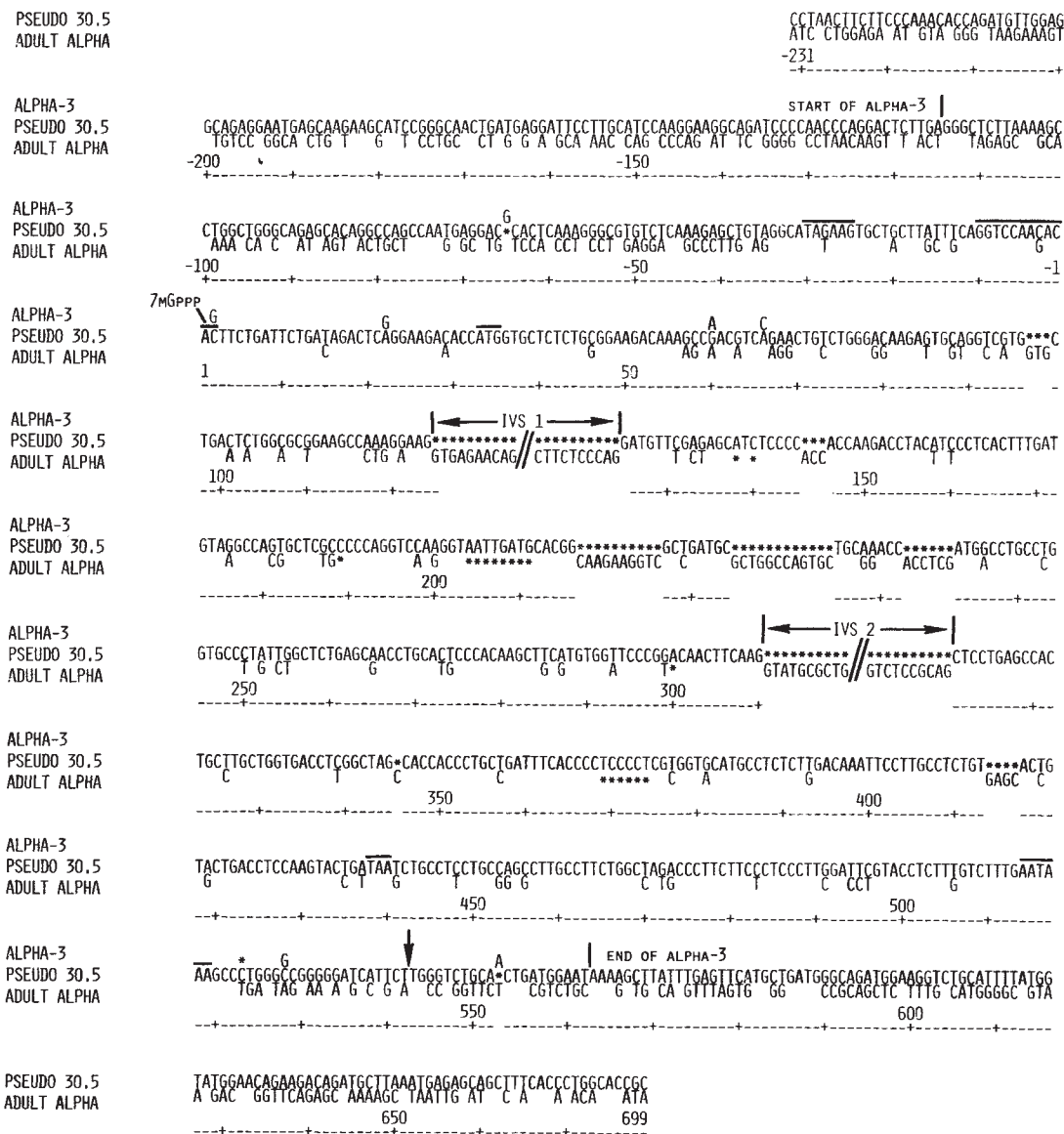


Fig. 1 Restriction maps and sequencing strategy for $\psi\alpha 30.5$. *a*, Map of the 4.7-kilobase pair *EcoRI* fragment of mouse DNA in clone Charon3AMm30.5 (refs 19,20). *b*, The *BamHI*/*BglII* fragment subcloned into the *BamHI* site of pBR322 before sequencing. *c*, The strategy used in sequencing $\psi\alpha 30.5$. The black areas show the region of the cloned DNA which is homologous to mouse α -globin mRNA. The horizontal arrows show the directions of sequencing. ³²P-end-labelling and DNA sequencing were by the Maxam and Gilbert procedures⁴² modified as described by Slightom *et al.*¹⁷. Other experimental procedures were essentially as described elsewhere¹⁷. bp, Base pairs.

*Present address: Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi 39216.

Fig. 2 The nucleotide sequence of the α -globin pseudogene, $\psi\alpha 30.5$. The nucleotide sequence of $\psi\alpha 30.5$ (pseudo 30.5, middle lines) is compared with the sequence of a productive adult α -globin gene⁹ (adult alpha, lower lines), and to the sequence of an equivalent pseudogene (alpha-3, upper lines) isolated from BALB/c mice and sequenced by Nishioka, Leder and Leder (personal communication). The sequence of $\psi\alpha 30.5$ is listed in full. Where either of the other sequences differs from $\psi\alpha 30.5$ the differing bases are shown; where they are identical only the sequence of $\psi\alpha 30.5$ is shown. Asterisks indicate that no bases are present at the indicated positions. The numbering system on the counting scale refers to $\psi\alpha 30.5$, with number 1 corresponding to the nucleotide homologous to the first nucleotide of α -globin mRNA. The intervening sequences, IVS 1 and IVS 2, of the adult α -globin gene are shown. Potentially important signals are overlined (see text). 7mGppp shows the position of the cap of adult α -globin mRNA. The arrow after nucleotide position 541 indicates the probable 3' end of α -globin mRNA (G. N. Pavlakis and J. N. Vournakis, personal communication). Note that the $\alpha 3$ sequence data do not cover nucleotide positions before -115 and after +561.



fragment in Charon3AMm30.5. Figure 1b shows the region subcloned into pBR322 before nucleotide sequencing, and Fig. 1c outlines the strategy used for nucleotide sequencing.

$\psi\alpha 30.5$ is related to an adult α -globin gene

Figures 2 and 3 present the nucleotide sequence for $\psi\alpha 30.5$ and compare it with the sequence of a productive α -globin gene from BALB/c mice⁹. The sequence of the equivalent pseudogene, $\alpha 3$, from BALB/c mice (Y. Nishioka, P. Leder and A. Leder, personal communication) is also given in Fig. 2. The nucleotide numbering system used refers to $\psi\alpha 30.5$ and assigns number 1 to the nucleotide in $\psi\alpha 30.5$ which is homologous to the first nucleotide of α -globin mRNA. The schematic comparison of the nucleotide sequences of $\psi\alpha 30.5$ and of the productive α -globin gene presented in Fig. 3 indicates where the two sequences are identical (dark areas) and at the same time emphasizes where they differ in length (triangles above and below the horizontal lines). The points where α -globin mRNA begins and ends are indicated by vertical arrows.

The first feature revealed by the sequence data of Fig. 2 and the schematic comparison of Fig. 3 is that $\psi\alpha 30.5$ is very closely related to the productive mouse α -globin gene. If we disregard additions and deletions, $\psi\alpha 30.5$ is identical in sequence to the mouse α -globin gene in 84% of the region coding for mRNA. When $\psi\alpha 30.5$ is compared with the

nucleotide sequence of the mouse β^{maj} globin gene¹¹ there is only 54% identity, which is not significantly different from the 58% identity found when the sequences of mouse α - and β^{maj} -globin genes are compared with each other. These comparisons show that $\psi\alpha 30.5$ is a member of the α -globin family rather than the β -globin family, which confirms our previous hybridization data²⁰. Also, $\psi\alpha 30.5$ seems more like the mouse adult α -globin gene than the mouse embryonic α -type globin gene, α ; the nucleotide sequence of $\psi\alpha 30.5$ has codons corresponding to an adult α -globin amino acid sequence rather than to the embryonic globin sequence²⁴ in nine discriminating positions which can be compared. Comparisons of the nucleotide sequence of $\psi\alpha 30.5$ with the sequences of the coding regions of all the adult α -globin genes for which sequence data are available show that $\psi\alpha 30.5$ is more closely related to the mouse adult α -globin gene⁹ (84% identity) than to either the human adult α -globin gene (74%) (S. Liehaber and Y. W. Kan, personal communication) or the rabbit adult α -globin gene²⁵ (72%).

$\psi\alpha 30.5$ lacks both intervening sequences

The second feature revealed by the data presented in Figs 2 and 3 is that $\psi\alpha 30.5$ completely lacks the two intervening sequences characteristic of productive globin genes. The absence of the intervening sequences in $\psi\alpha 30.5$ is clean, in that the nucleotide sequence immediately 5' and 3' to the

intervening sequences in the adult α -globin gene⁹ are contiguous in $\psi\alpha 30.5$; the nucleotide sequence in these regions of $\psi\alpha 30.5$ is exactly the same as that seen in fully processed mouse α -globin mRNA (ref. 26, our unpublished observations, and G. N. Pavlakis and J. N. Vournakis, personal communication). It is as if the intervening sequences had never been there or had been perfectly excized.

$\psi\alpha 30.5$ is an α -globin pseudogene

Inspection of the nucleotide sequence of $\psi\alpha 30.5$ shows that it has several signals which might be expected in a productive α -globin gene. There is a sequence (TAGAAG) at nucleotide positions -30 to -25 comparable to the version of the Pribnow-like box²⁷ found in the productive α -globin gene (TATAAG) and thought to be important in the initiation of transcription⁹. There is a sequence (GGTCCAACACAC) at the nucleotide positions -10 to +2 comparable to the (GTTCGTCCTCAC) sequence postulated by Ziff and Evans²⁸ to be important in the 'capping' of mRNA. The expected translation initiation codon (ATG) and chain terminator codon (TAA) occur at the appropriate nucleotide positions (33-35 and 438-440). Finally, there is a sequence (AATAAA) at positions 514-519 which is identical to the so-called poly(A) addition signal (AATAAA) of Proudfoot and Brownlee²⁹. We do not know whether these regions could provide adequate signals for all stages of normal transcription and translation, as the absolute requirements of these signals are not known. However, it is quite remarkable that, although the region of strong homology between $\psi\alpha 30.5$ and the productive α -globin gene is extensive enough to include all these potential signals, beyond the outside signals the homology is virtually non-existent (see Figs 2, 3). Thus, on the 5' (upstream) side of the two genes, the strong homology extends to nucleotide position -34, a few bases upstream from the Pribnow-like box²⁷. On the 3' (downstream) side of the two genes, the strong homology ends at nucleotide position 522, a few bases downstream from the Proudfoot-Brownlee sequence²⁹. The strong homology does not extend to the 3' end of α -globin mRNA, which is close to nucleotide position 541 (G. N. Pavlakis and J. W. Vournakis, personal communication).

Even if $\psi\alpha 30.5$ could be transcribed into RNA which was then processed to yield a functional message, it could not code for a functional α -globin polypeptide. Figure 4 shows the amino acid sequence which corresponds to the nucleotide sequence of $\psi\alpha 30.5$ on the assumption that it is translated in the same reading frame as the productive α -globin gene. The first 34 of these amino acid residues are very similar to those found in α globins³⁰, but a frameshift after amino acid residue 34, due to the addition in $\psi\alpha 30.5$ of two bases, results in three chain terminator codons (one of each variety) shortly thereafter, and also causes the coded amino acid sequence to become unrelated (in *italics*) to α globin. Other frameshifts, due to the addition or deletion of non-integral triplets of nucleotides, occur elsewhere in the sequence of $\psi\alpha 30.5$, and restore it to the α -globin reading frame briefly from residues 53

to 56 and again from residues 134 to 140.

Because $\psi\alpha 30.5$ is clearly related in nucleotide sequence to an adult α -globin gene (Figs 2, 3), but could not code for a functional α -globin polypeptide (Fig. 4), we call it an α -globin pseudogene. This terminology does not exclude the possibility that $\psi\alpha 30.5$ codes for a non-globin polypeptide. For example, it is conceivable (although unlikely) that $\psi\alpha 30.5$ codes for a polypeptide homologous to α globin in its N-terminal and C-terminal regions but shorter because of the excision of a currently unrecognized intervening sequence covering the chain terminators caused by the frame shifts.

The α -globin pseudogenes in two mouse strains are almost identical

Comparison of the nucleotide sequences of $\psi\alpha 30.5$ and the equivalent gene, $\alpha 3$, from BALB/c mice (Y. Nishioka, P. Leder and A. Leder, personal communication) shows (Fig. 2) that they are almost identical (>99% identity excluding deletions and additions). Both lack the two intervening sequences, and both are pseudogenes in the sense that frameshifts would prevent them from producing functional globin polypeptides. The number of differences between the α -globin pseudogenes from the two strains is close to the level attributable to errors in sequencing; thus, relative to $\psi\alpha 30.5$, the BALB/c pseudogene has five base substitutions and three single base additions or deletions.

How did the α -globin pseudogene $\psi\alpha 30.5$ evolve?

Any evolutionary history of the α -globin pseudogene, $\psi\alpha 30.5$, must take into account the following key observations: (1) all characterized productive globin genes of both α - and β -type have two intervening sequences at homologous places in their coding regions; (2) $\psi\alpha 30.5$ lacks the intervening sequences cleanly; (3) $\psi\alpha 30.5$ is much more closely related to an adult α -globin gene than to the β -type genes, and more to the mouse than to the human or rabbit adult α -globin genes; (4) $\psi\alpha 30.5$ could not code for a functional globin polypeptide.

The first of these key observations has led to the suggestion by Leder *et al.*⁸ that two intervening sequences became evolutionarily fixed in primitive globin genes before the α - and β -type genes diverged. If this is correct, three general types of evolutionary history can be suggested for $\psi\alpha 30.5$. Each has problems, and we have no means of excluding any of them, although we favour the second.

First, $\psi\alpha 30.5$ could be the present-day descendant of an old duplicate of a primitive globin gene which had never had intervening sequences. In this case, there are two important residual problems. Why did the pseudogene persist for so long, especially after it became unable to produce a functional globin chain? What mechanism or selective pressures caused it to co-evolve with the productive globin genes to the extent that it is recognizably related to an α -globin gene and particularly to a mouse adult α -globin gene? The simplest explanation for the evolutionary persistence of pseudogenes is

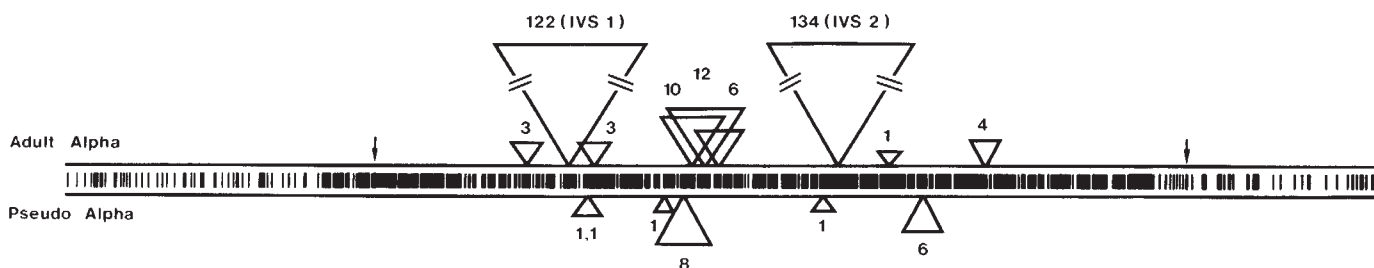


Fig. 3 A schematic comparison of the nucleotide sequences of the productive adult α -globin gene and of the pseudogene $\psi\alpha 30.5$. The central two parallel horizontal lines represent regions of the two genes which are directly comparable. Each nucleotide identical in the two sequences is represented by a vertical stroke between these two horizontal lines; therefore, dark regions represent segments of extensive nucleotide homology. The triangles show places where the two genes differ in their lengths, and indicate the number of nucleotides in each length difference. (The triangle labelled 1,1 represents two single base additions separated by one base.) Triangles are drawn above the parallel lines when the adult α gene is longer, and below the lines when pseudogene $\psi\alpha 30.5$ is longer. Two vertical arrows show where α -globin mRNA begins and ends.

1.....5.....10.....15.....21/23.....26
 (Met)ValLeuSerAlaGluAspLysAlaAspValArgThrValTrpAspLysSerAlaGlyArgAlaAspSerGlyAla
 27.....35
 GluAlaLysGlyArgMetPheGlySerIleSerProProArgProThrSerLeuThrLeuMet * AlaSerAlaArg
 54.....57
 ProGlnValGlnGlyAsn * CysThrGly * CysCysLysProTrpProAlaTrpCysProIleGlySerGluGln
 ProAlaLeuProGlnAlaSerCysGlySerArgThrThrSerSerSer * AlaThrAlaCysTrp * ProArgLeu
 134...136
 AlaProProCys * PheTyrProSerProArgGlyAlaCysLeuSer * GlnIleProCysLeuCysThrValLeu
 137.....140
 ThrSerLysTyr * *

Fig. 4 The amino acid sequence corresponding to the nucleotide sequence of $\psi\alpha 30.5$ on the assumption that it is translated in the same reading frame and from the same initiator codon as the productive α -globin gene. The numbered scales show amino acid residue numbers in α globins. In frame α -globin amino acids are underlined. Amino acids in a different reading frame from α globin, or that have never been seen in an α globin, are italicized. Asterisks indicate chain terminator codons.

that they serve some important function, even though they are unable to code for globin polypeptides. The similarities between $\psi\alpha 30.5$ and the adult α gene might then be a consequence of strong selective pressures keeping their sequences from diverging, or it might be due to the existence of mechanisms other than selection for maintaining similarities between related genes within a species. Recent nucleotide sequence data from our laboratory suggest that the human G_γ and A_γ fetal globin genes have remained very similar as a result of intergenic exchanges of DNA occurring during recombinational events comparable to gene conversion¹⁷.

Second, $\psi\alpha 30.5$ could be the descendant of a much more recent duplicate of a mouse adult α -type globin gene which had both intervening sequences. In this case, the important residual problem is explaining how they were then lost so exactly. The exact loss of both intervening sequences by random deletions at the DNA level would require an improbable series of events the selective advantages of which are individually difficult to imagine. It is easier to imagine that a molecular mechanism exists able to remove intervening sequences exactly from DNA in a fashion comparable to that used at the RNA level. Evidence in support of such a mechanism is provided by recent data on the preproinsulin genes of chicken³¹, rat^{9,32} and human³³. All three species have a preproinsulin gene, with two intervening sequences in homologous positions. However, the rat, unlike the chicken and human, has an additional functional preproinsulin gene containing only the smaller first intervening sequence. Bell *et al.*³³ and Perler *et al.*³¹ postulate that the two rat genes arose from a duplication, after which the larger intervening sequence was eliminated from one of the genes.

Third, a completely different type of evolutionary scheme can be imagined whereby $\psi\alpha 30.5$ was recently derived from mouse adult α -globin mRNA. It is conceivable that a reverse transcript of a mouse α -globin mRNA was reintroduced into the genome, perhaps by recombination with a pre-existing α -globin gene. We know of no precedents for this type of mechanism except those related to the retroviruses, although the general concept was strongly advocated by Temin³⁴ in his provirus hypothesis. This type of evolutionary scheme faces a major problem in accounting for the introduction of the reverse transcript into the germ line, because globin mRNA is normally produced in erythropoietic cells belonging to a cell lineage that is developmentally and temporally separated from the germ-cell lineage. Although the reverse transcript scheme is in general compatible with the sequence data for $\psi\alpha 30.5$, special explanations would be required to account for the fact that the extent of the homology between the $\psi\alpha$ and α genes

does not coincide exactly with the mRNA boundaries.

Have globin pseudogenes any function?

$\psi\alpha 30.5$ is not unique in being a globin pseudogene. Maniatis and his collaborators have isolated several globin-related DNA sequences which cannot be identified with known globin polypeptides. In rabbits a gene, designated β_2 , is found 5' to the productive adult β gene²¹; it has limited sequence homology to adult and embryonic β -globin probes, it lacks a detectable mRNA transcript in the erythroid tissues examined (anaemic blood and bone marrow), and its larger intervening sequence does not hybridize to the larger intervening sequence of the productive adult β -globin gene²². Similar sequences ($\psi\beta_1$ and $\psi\beta_2$) which do not correspond to any of the β -type globin polypeptides have been identified in humans²³. $\psi\beta_1$ is on the 5' side of the productive adult β -type genes, and $\psi\beta_2$ is on the 5' side of the embryonic ϵ gene. A gene, $\psi\alpha_1$, which does not code for any known globin polypeptide, has also been identified on the 5' side of the two productive human adult genes¹⁸. Thus, each of these unusual genes is located in a comparable position on the 5' side of productive globin genes. (Note, however, that the position of $\psi\alpha 30.5$ in the mouse genome is not yet known.)

Although the human sequences $\psi\beta_1$ and $\psi\beta_2$ have not been studied in detail, the nucleotide sequences of both the rabbit gene β_2 (E. Lacy and T. Maniatis, personal communication) and the human gene $\psi\alpha_1$ (N. Proudfoot and T. Maniatis, personal communication) have been found to differ from the sequences of the corresponding productive globin genes in several ways which would make them incapable of coding for functional globin polypeptides. They are consequently also pseudogenes, although they differ from the pseudogene $\psi\alpha 30.5$ in having intervening sequences. However, their intervening sequences are unusual in ways which would be expected to prevent them from being spliced out in the normal manner (E. Lacy, N. Proudfoot and T. Maniatis, personal communication). Thus, there are pseudogenes having unusual (or absent) intervening sequences associated with productive α - and β -type globin genes in all species for which detailed information is available. This leads us to conclude that they are probably important, and it is attractive to consider them as controlling, in some fashion, the productive globin genes. We think it unlikely that the globin pseudogenes are dead genes without function.

How might pseudogenes control productive genes?

We are investigating the possibility that pseudogenes might be 'diverting genes' able to modulate the expression of productive genes by diverting transcription into a degradative pathway. This possible mode of action of pseudogenes has two prerequisites: first, that each globin pseudogene and its related productive genes are members of the same transcriptional unit, although not necessarily of the same transcript; second, that a mechanism exists to select which portion of this unit is used. If the pseudogene portion is selected for transcription from the unit, the transcript would be degraded because the pseudogene lacks normal intervening sequences. If a productive gene portion is selected, processing of the transcript, transport out of the nucleus and translation could occur normally. Examples of processes similar to those envisaged here can be found in the literature: in adenovirus type 2, different portions of the late transcript are used in different mRNAs³⁵; in a globin-SV40 system, transcripts not associated with normal intervening sequences do not yield globin polypeptides^{36,37}.

A second possibility is that $\psi\alpha 30.5$ is an 'antigene', by which we mean that $\psi\alpha 30.5$ is transcribed from the opposite (complementary) strand of DNA from that used in the productive gene. Such an anti-transcript would have the potential for forming an RNA-RNA heteroduplex with the productive gene transcript in a manner similar to that proposed by Davidson and Britten³⁸ for the transcripts of

some repetitive DNAs. The resulting structure, with a form similar to that shown in Fig. 3, might function as a positive controlling element in erythroid tissues, or as a negative element in non-erythroid tissues. (This idea does not exclude the possibility that pseudogene anti-transcripts form ternary complexes with the transcripts of the corresponding productive genes and the small stable nuclear RNAs recently implicated in RNA processing by Lerner *et al.* and Rogers and Wall^{39,40}.)

Preliminary experiments aimed at exploring the transcriptional status of the $\psi\alpha 30.5$ pseudogene have been done in erythroid tissue. We prepared ³²P-end-labelled fragments from $\psi\alpha 30.5$ DNA which could be used as specific probes for the 'sense' and 'antisense' transcripts of the $\psi\alpha 30.5$ pseudogene. We tested, by the Berk and Sharp procedure⁴¹, the ability of total RNA from mouse fetal liver cells (an erythroid tissue) to form RNA-DNA hybrids capable of protecting these probes from S₁ nuclease. We have been unable in these preliminary experiments to find evidence for significant protection by the fetal liver RNA of either the sense or the antisense probes derived from $\psi\alpha 30.5$, using conditions which allow easy detection of globin precursor RNA (A. J.

Kinniburgh, personal communication). Thus, if the pseudogene is transcribed in erythroid tissues, its available transcript must be at a steady-state level lower than that of globin precursor RNA. This result agrees with that already cited for the rabbit pseudogene β_2 (ref. 22).

The general hypothesis that pseudogenes control the productive genes in some fashion, nevertheless, remains attractive and we are investigating the hypothesis further, including tests in non-erythroid tissues. Certainly, the widespread occurrence of globin pseudogenes argues strongly for their functional importance.

We thank Drs Y. Nishioka, A. Leder, P. Leder, N. Proudfoot and T. Maniatis for sequence data before publication, Natalie Borenstein and John Devereux for technical assistance, Dr J. L. Slightom for advice in the DNA sequencing, and Drs A. J. Kinniburgh and J. Ross for advice and help in the experiments testing for transcription of $\psi\alpha 30.5$. This work was supported by NIH grants AM20120 and GM20069 to O.S., and GM21812 to Dr F. R. Blattner, whom we thank for the shared use of laboratory equipment. The recombinant DNA experiments were carried out in accordance with NIH guidelines.

Received 25 March; accepted 2 May 1980.

1. Breathnach, R., Mandel, J. L. & Chambon, P. *Nature* **270**, 314-319 (1977).
2. Brač, C. & Tonegawa, S. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5652-5656 (1977).
3. Leder, P. *et al. Cold Spring Harb. Symp. quant. Biol.* **42**, 915-920 (1977).
4. Jeffreys, A. J. & Flavell, R. A. *Cell* **12**, 1097-1108 (1977).
5. Lomedico, P. *et al. Cell* **18**, 545-558 (1979).
6. Crick, F. *Science* **204**, 264-271 (1979).
7. Abelson, J. A. *Rev. Biochem.* **48**, 1035-1069 (1979).
8. Leder, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 6187-6191 (1978).
9. Nishioka, Y. & Leder, P. *Cell* **18**, 875-882 (1979).
10. Tilghman, S. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 4406-4410 (1977).
11. Konkel, D. A., Tilghman, S. M. & Leder, P. *Cell* **15**, 1125-1132 (1978).
12. Tiemeier, D. C. *et al. Cell* **14**, 237-245 (1978).
13. Konkel, D. A., Maizel, J. V. Jr & Leder, P. *Cell* **18**, 865-873 (1979).
14. van den Berg, J. *et al. Nature* **276**, 37-44 (1978).
15. Dodgson, J. B., Strommer, J. & Engel, J. D. *Cell* **17**, 879-887 (1979).
16. Lawn, R. M., Fritsch, E. F., Parker, R. C., Blake, G. & Maniatis, T. *Cell* **15**, 1157-1174 (1978).
17. Slightom, J. L., Blechl, A. E. & Smithies, O. *Cell* (in the press).
18. Lauer, J., Shen, C.-K. J. & Maniatis, T. *Cell* **20**, 119 (1980).
19. Blattner, F. R. *et al. Science* **202**, 1279-1284 (1978).
20. Smithies, O. *et al. Science* **202**, 1284-1289 (1978).
21. Lacy, E., Hardison, R. C., Quon, D. & Maniatis, T. *Cell* **18**, 1273-1283 (1979).
22. Hardison, R. C. *et al. Cell* **18**, 1285-1297 (1979).
23. Fritsch, E. F., Lawn, R. M. & Maniatis, T. *Cell* **19**, 959-972 (1980).
24. Melderis, H., Steinheider, G. & Ostertag, W. *Nature* **250**, 774-776 (1974).
25. Heindel, H. C., Liu, A., Paddock, G. V., Studnicka, G. M. & Salser, W. A. *Cell* **15**, 43-54 (1978).
26. Pavlakis, G. N. *et al. Cell* **19**, 91-102 (1980).
27. Pribnow, D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 784-788 (1975).
28. Ziff, E. B. & Evans, R. M. *Cell* **15**, 1463-1475 (1978).
29. Proudfoot, N. J. & Brownlee, G. G. *Nature* **263**, 211-214 (1976).
30. Dayhoff, M. O. (ed.) in *Atlas of Protein Sequence and Structure* Vol. 5, Suppl. 2, 191-223 (National Biomedical Research Foundation, Washington, DC, 1976).
31. Perler, F. *et al. Cell* (in the press).
32. Cordell, B. *et al. Cell* **18**, 533-543 (1979).
33. Bell, G. I. *et al. Nature* **284**, 26-32 (1980).
34. Temin, H. M. A. *Rev. Genet.* **8**, 155-177 (1974).
35. Nevins, J. R. & Darnell, J. E. *Cell* **15**, 1477-1493 (1978).
36. Hamer, D. H., Smith, K. D., Boyer, S. H. & Leder, P. *Cell* **17**, 725-735 (1979).
37. Hamer, D. H. & Leder, P. *Cell* **17**, 737-747 (1979); **18**, 1299-1302 (1979).
38. Davidson, E. H. & Britten, R. J. *Science* **204**, 1052-1059 (1979).
39. Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. I. & Steitz, J. A. *Nature* **283**, 220-224 (1980).
40. Rogers, J. & Wall, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1877 (1980).
41. Berk, A. J. & Sharp, P. A. *Cell* **12**, 721-732 (1977).
42. Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-564 (1977).

Proximity of mRNA 5'-region and 18S rRNA in eukaryotic initiation complexes

Kunio Nakashima*, Edward Darzynkiewicz† & Aaron J. Shatkin

Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Messenger RNA was covalently linked to 18S ribosomal RNA in eukaryotic 40S and 80S initiation complexes by photoreaction with an RNA cross-linking agent, 4'-substituted psoralen. The sites of interaction of the mRNA capped 5'-region included some 3'-ends of 18S rRNA.

AN early step in protein synthesis is the binding of mRNA to ribosomal small subunits. The resulting initiation complexes in prokaryotes apparently are stabilized by base pairing between the 3'-terminal sequence of 16S ribosomal RNA, ...CCUCCUUA, and a purine-rich nucleotide stretch located in mRNA on the 5'-side of the AUG initiator codon¹⁻³. The suggestion that mRNA-rRNA interactions are similarly involved in the initiation of eukaryotic translation⁴ has been a topic of considerable speculation⁵⁻⁹. Comparative analyses have demonstrated a highly conserved 3'-terminal sequence in the small rRNAs of *Escherichia coli*¹, *Bacillus stearothermophilus*¹⁰ and maize chloroplast¹¹ as well as in

yeast¹², slime mould, wheat, silkworm, mouse⁵, rat¹³ and rabbit¹⁴ but the CCUCC sequence implicated in prokaryotic initiation is absent from eukaryotic 18S rRNA. Thus, conservation of the 3' sequence may be related to rRNA processing or events other than initiation of protein synthesis. However, some eukaryotic viral¹⁵ and cellular¹⁶ mRNAs contain within their noncoding 5' region nucleotide sequences complementary to the 3' end of 18S rRNA and consequently at least could contribute to stable initiation complex formation.

Psoralens are tricyclic photoreactive compounds that intercalate into nucleic acids. Irradiation of polynucleotide-bound psoralen with long wavelength UV light produces adducts with pyrimidine bases. Because psoralens are bifunctional agents, they can also form cross-links between interacting pyrimidine-containing nucleotide sequences¹⁷. 4'-Substituted derivatives of psoralen with improved

*Present address: Department of Biochemistry, Yamagata University School of Medicine, Zao-Iida, Yamagata, Japan 990-23.

†Permanent address: Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Warsaw, Poland.

photoreactivity have been synthesized recently and have already proved useful for investigating genome structure-function relationships in RNA viruses¹⁸. For example, photoreaction of reovirus with 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) produced RNA-RNA cross-links between the two complementary strands comprising the viral genome and inactivated its template function for the virion-associated RNA polymerase¹⁹. These findings suggested that AMT photoreaction would be useful for probing other types of nucleoprotein complexes, such as those formed between mRNA and ribosomes during initiation of protein synthesis.

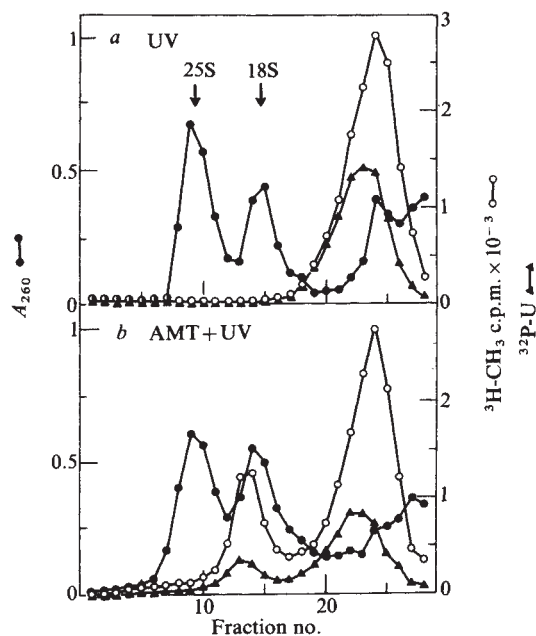


Fig. 1 Cross-linking of reovirus mRNA to 18S rRNA in wheat germ 40S initiation complexes. Reovirus mRNA radiolabelled with ^3H -methyl in the 5'-terminal cap, $\text{m}^7\text{GpppG}^{\text{m}}$ and internally with ^{32}P -UMP was synthesized *in vitro* as described previously⁴³. The viral mRNA was nicked to fragments sedimenting at 4-6S by incubation in 50 mM NH_4HCO_3 , pH 10, at 98°C for 3 min. The fragments were used to form 40S initiation complexes by incubation in wheat germ translating extracts in the presence of 0.2 mM sparsomycin to block elongation and 0.4 mM guanosine-5'-(β,γ -methylene)triphosphate to decrease 60S subunit joining to 40S-mRNA complexes²³. Complexes were isolated by centrifugation for 4 h and 189,000g in the SW40 rotor in 10-30% glycerol gradients containing 10 mM Tris-HCl (pH 7.4), 0.75 mM Mg acetate and 70 mM KCl²⁰. Fractions containing 40S initiation complexes (which also included free 60S subunits) were combined and irradiated with long wavelength UV light for 20 min at 0°C in the absence (a) and presence (b) of 2.8×10^{-4} M 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT) as described previously⁴⁴. The irradiated mixtures were incubated at 37°C for 1 h with proteinase K (1 mg ml⁻¹) and 0.5% SDS, and the RNA was extracted with water-saturated phenol and analysed by centrifugation (SW-41, 131,000g, 16 h, 5°C) in 10-30% glycerol gradients containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1 mM EDTA. Fraction aliquots were counted in Aquasol (NEN) after determining the $A_{260 \text{ nm}}$.

We have used this approach to look for RNA-RNA interactions between the ends of radiolabelled mRNA and rRNA in eukaryotic initiation complexes.

Cross-linking of reovirus mRNA and 18S rRNA in wheat germ 40S and 80S initiation complexes

Most eukaryotic mRNAs are monocistronic. During initiation of translation ribosomes bind to mRNA at or near the 5' cap

and re-position, perhaps by a scanning mechanism, at the 5'-proximal AUG codon to start polypeptide synthesis⁶. Previous studies with reovirus mRNA and mRNA fragments labelled with ^3H -methyl in the 5' cap, $\text{m}^7\text{GpppG}^{\text{m}}$, and at internal sites with ^{32}P have demonstrated that capped molecules are selectively bound to ribosomes²⁰⁻²². Thus when ^3H -methyl, ^{32}P -UMP labelled, alkali-fragmented reovirus mRNA (chain length ~200 nucleotides) was incubated in wheat germ cell-free translating extract in conditions of 40S initiation complex formation, 32% of the ^3H and 4% of the ^{32}P was bound to ribosomal small subunits and sedimented in a glycerol gradient in a position close to 40S subunits; similar results have been described previously^{23,24}. To determine whether mRNA and rRNA were closely apposed in these complexes, pooled fractions from the 40-60S region of the gradient were irradiated at $\lambda_{352 \text{ nm}}$ in the presence and absence of the RNA cross-linking agent, AMT, and the RNA was extracted from the initiation complexes and analysed by gradient sedimentation. As shown in Fig. 1a, initiation complexes irradiated in the absence of AMT yielded labelled mRNA

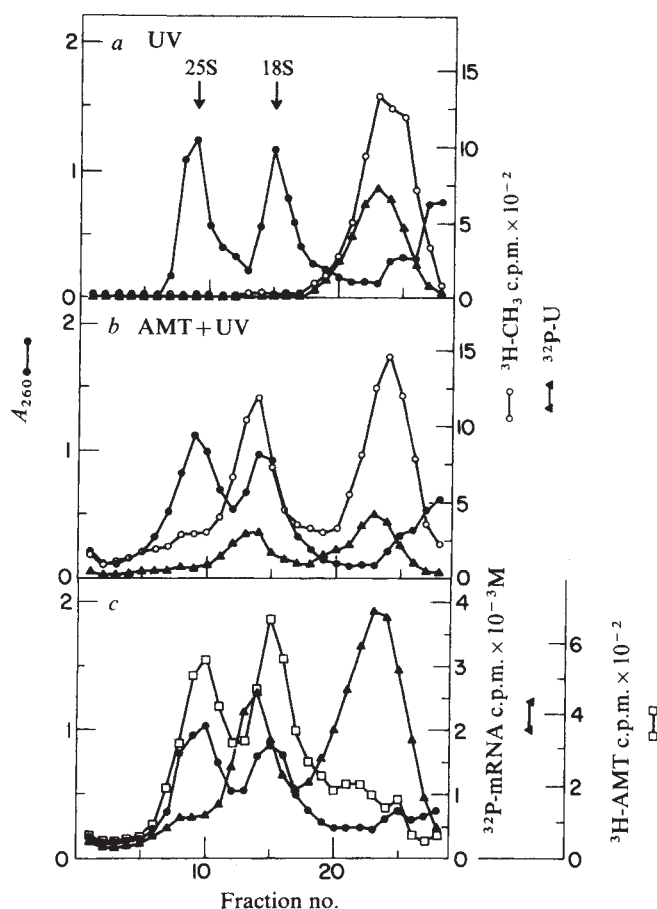


Fig. 2 Cross-linking of reovirus mRNA to 18S rRNA and covalent binding of ^3H -AMT to rRNAs in wheat germ 80S initiation complexes. Radiolabelled reovirus mRNA fragments were incubated as in Fig. 1 except that mixtures contained 0.2 mM GTP in place of guanosine-5'-(β,γ -methylene)triphosphate to obtain 80S complexes. Complexes containing double-labelled fragments were isolated by glycerol gradient centrifugation and UV-irradiated in the absence (a) or presence (b) of 2.8×10^{-4} M AMT. For c, fragments were prepared from mRNA synthesized in the presence of all four [$\alpha\text{-}^{32}\text{P}$]labelled ribonucleoside triphosphates and unlabelled S-adenosylmethionine, and the incubation mixture, rather than isolated initiation complexes, was irradiated for 20 min in 1.4×10^{-4} M ^3H -labelled AMT (specific activity 1.4×10^5 c.p.m. μg^{-1}). RNA was extracted and analysed by glycerol gradient centrifugation as described in the legend to Fig. 1.

fragments that sedimented at the top of the gradient, well-separated from the rRNAs. By contrast, complexes that were irradiated in the presence of AMT yielded a significant portion of both the ^3H (28%) and ^{32}P (25%) sedimenting in a position slightly faster than the small ribosomal RNA (Fig. 1b).

Like 40S initiation complexes, 80S complexes irradiated with AMT also yielded a large fraction (38%) of the radioactive mRNA fragments cross-linked to 18S rRNA (Fig. 2a,b). Apparently there was little interaction of mRNA fragments with the large rRNA in initiation complexes. However, the rRNAs in both ribosomal subunits were accessible to psoralen photoreaction. As shown in Fig. 2c, in conditions which resulted in cross-linking of ^{32}P -labelled mRNA fragments to 18S rRNA, both rRNAs formed ^3H -labelled AMT adducts; psoralen average molar ratios were 0.8 and 1.5 for the small and large rRNAs, respectively.

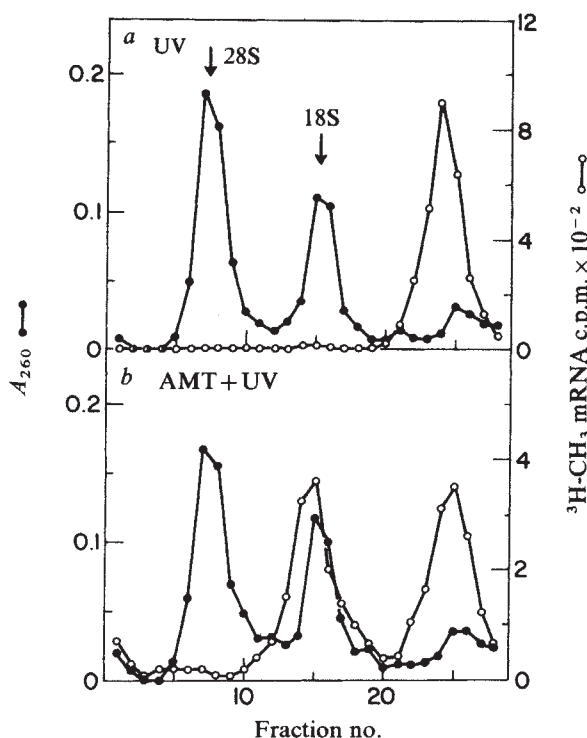


Fig. 3 Cross-linking of vesicular stomatitis virus mRNA to 18S rRNA in reticulocyte 80S initiation complexes. ^3H -methyl-labelled viral mRNA containing all radioactivity in the 5'-terminal cap, $\text{m}^7\text{GpppA}^{\text{m}}$ was synthesized *in vitro* in the presence of $2\mu\text{M}$ *S*-adenosyl-L-[Me- ^3H]methionine (specific activity 70Ci mmol^{-1})⁴⁵. Rabbit reticulocyte lysates (treated with haemin)⁴⁶ were used to form 80S initiation complexes as in Fig. 2. Gradient fractions containing complexes were combined, irradiated in the absence (a) or presence (b) of $2.8 \times 10^{-4}\text{M}$ AMT, and the extracted RNA analysed as described for Fig. 1.

Results essentially the same as those in Figs 1 and 2 were obtained with intact mRNA and when sedimentation analyses were done in denaturing conditions in 99% Me_2SO -sucrose gradients, although the ribosomal and mRNAs were less well resolved. With either intact mRNA or fragments, interaction with 18S rRNA was dependent on initiation complex formation. Cross-linked hybrid molecules were not obtained by AMT photoreaction of a mixture of mRNA and purified rRNAs. Furthermore, cross-linking of ^3H -methyl, ^{32}P -AMP-labelled mRNA to rRNA was not observed following AMT photoreaction of incubation mixtures that were complete except for the absence of ATP which is required for mRNA binding to ribosomes^{25,26}.

Interaction of 18S rRNA with other mRNAs in rabbit reticulocyte and wheat germ initiation complexes

Various other 5'-terminally labelled viral mRNAs including *in vitro* transcripts synthesized by the virion-associated RNA polymerases of vesicular stomatitis (VSV), influenza and cytoplasmic polyhedrosis (CPV) viruses and genome RNAs of tobacco mosaic (TMV) and satellite tobacco necrosis (STNV) viruses were tested. Each of these RNAs interacted with the 18S rRNA in wheat germ initiation complexes as detected by AMT photoreaction (data not shown). In addition, 40S and 80S initiation complexes formed in rabbit reticulocyte lysates gave results similar to those obtained in the wheat germ system. For example, when initiation complexes of reticulocyte 80S ribosomes and fragmented VSV mRNA containing 5'-terminal ^3H -methyl-labelled $\text{m}^7\text{GpppA}^{\text{m}}$ were photoreacted with AMT, 47% of the ribosome-bound radioactivity cross-linked to, and sedimented with the 18S rRNA (Fig. 3).

Capped ribopolymers, synthesized by incubating polynucleotide phosphorylase, ribonucleoside diphosphates and ^3H -methyl- $\text{m}^7\text{GpppG}^{\text{m}}$ -C in primer-dependent conditions, have proved useful for studies of structural and compositional effects on mRNA binding to ribosomes²⁷. Capped poly(U) forms only 40S initiation complexes with wheat germ ribosomes while capped poly(A,U) associates with 80S monosomes²⁷. However, as observed with natural mRNA, AMT photoreaction of the 40S-capped poly(U) complexes or of the capped poly(A,U)-40S and 80S complexes resulted in polymer cross-linking to 18S rRNA only (data not shown).

Sites of cross-linking of 18S rRNA to 5' end of mRNA

Initially we attempted to test whether the 3' region of 18S rRNA interacts with mRNA during initiation by using prelabelled ribosomes but were unsuccessful in radiolabelling the 3' terminus of rRNA *in situ* by incubating wheat germ extracts with ^{32}P -pCp and RNA ligase²⁸. Instead we used the same technique to label purified, cross-linked hybrids of 18S rRNA and mRNA fragments isolated from AMT-photoreacted wheat germ 80S initiation complexes. They were formed with 5'- ^3H -methyl-labelled reovirus mRNA that had been treated at pH 10 before ribosome binding to produce mRNA fragments containing a 3'-terminal phosphate. As a consequence, ^{32}P -pCp-labelling of the cross-linked RNA-RNA complexes was apparently limited to free hydroxyl groups at the 3' end of the rRNA strands. The radiolabelled sample, which included an excess of 18S rRNA from ribosomes that did not bind mRNA in addition to rRNA-mRNA complexes, was digested with RNase T_1 . The resulting oligonucleotide fragments were loaded onto a column of acetylated dihydroxyboryl (DBAE)-cellulose in conditions that result in the selective binding of those oligonucleotides that contain 2',3'-*cis*-diols²⁹. Since the ^{32}P was present as ^{32}P -pCp at the 3' end of rRNA (and T_1 RNase digestion results in oligonucleotides with a 3'-terminal phosphate), 99% of the ^{32}P -labelled fragments did not bind to the resin. The ^3H radioactivity, however, bound to the extent of 78% as expected for mRNA fragments containing 5'-terminal $\text{m}^7\text{G}^5'\text{ppp}^5'\text{G}^{\text{m}}$, that is, a 2',3'-*cis*-diol in the 5'-linked m^7G (ref. 30). The bound oligonucleotides were eluted and rechromatographed on DBAE-cellulose. There was quantitative binding of the ^3H -labelled material, and more than 70% of the ^{32}P -labelled oligonucleotides were retained on the resin in the second pass (Fig. 4a), presumably due to cross-linking to capped mRNA fragments since in the absence of AMT photoreaction there was little ^{32}P rebinding.

The DBAE-bound sample was eluted and analysed by electrophoresis in a 20% polyacrylamide gel in denaturing conditions in 7M urea. Although the capped oligonucleotides released from at least six of the reovirus mRNA species by RNase T_1 digestion are 8 to 10 nucleotides long³¹, the ^3H -labelled cross-linked sample migrated as a broad peak of

chain length ~15 to ~35 nucleotides as estimated from the position of marker dyes, bromophenol blue and xylene cyanol (Fig. 4b). The ^{32}P -labelled oligonucleotides were resolved into a fraction that co-migrated with the peak of ^3H and a larger, more heterogeneous component near the xylene cyanol dye. On the basis of the known 3'- and 5'-terminal sequences of rRNA and reovirus mRNAs, RNase T_1 treatment would release from 3'- ^{32}P -pCp-labelled wheat germ 18S rRNA⁵ the radioactive oligonucleotide, AUCAUUG- ^{32}P and from a mixture of ^3H -methyl-labelled reovirus mRNAs, several 5'-capped A,U-rich oligonucleotides of chain length 8–10 nucleotides³¹. Cross-linking by AMT of the rRNA 3' end to mRNA 5' fragments would result in hybrids of minimum size ~15–17 nucleotides, a value corresponding reasonably well to

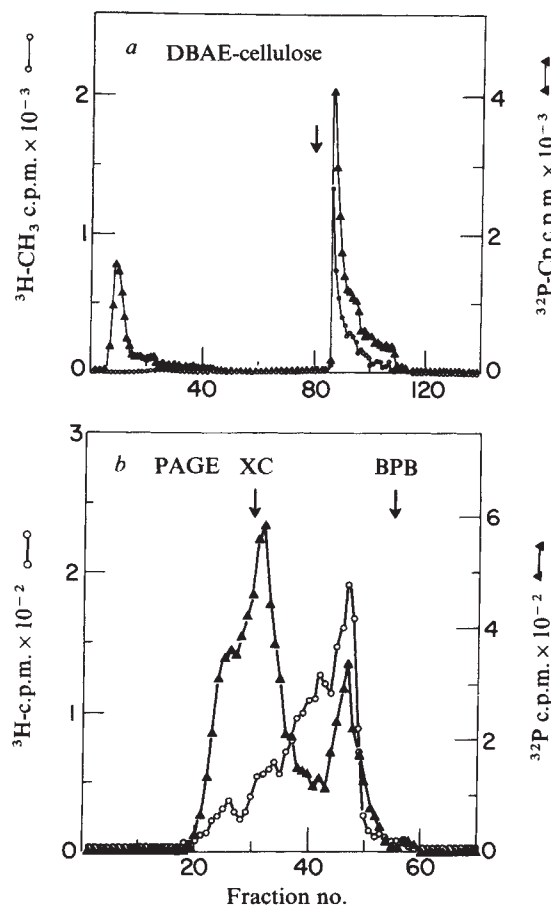


Fig. 4 Analysis of RNase T_1 digest of 5' ^3H -methyl labelled reovirus mRNA cross-linked to 3'- ^{32}P -labelled 18S rRNA. Wheat germ 80S initiation complexes were isolated, photoreacted with AMT and the cross-linked complexes of mRNA photoreacted with 18S rRNA were obtained as in Fig. 1. rRNA in the complexes was radiolabelled at the 3' end by incubation with ^{32}P -pCp and T_4 RNA ligase²⁸. After digestion with RNase T_1 (200 μg RNA, 100 units T_1 in 0.5 ml 20 mM Tris-HCl buffer pH 8 containing 0.1 mM EDTA) at 37°C for 1 h, the digest was added to 2 ml of buffer A³⁰ and applied to a column (0.6 $\times$ 5 cm) of acetylated dihydroxyboryl-substituted cellulose (DBAE-cellulose). After extensive washing with buffer A, bound material was eluted with 1 M sorbitol in buffer A and precipitated by addition of 3 volumes of ethanol and standing overnight at -20°C. The precipitated RNA was recovered by centrifugation and rechromatographed on DBAE-cellulose (a). Aliquots of fractions were counted in Aquasol. The RNA in fractions 85–115 (arrow indicates start of sorbitol elution) was recovered by ethanol precipitation and analysed by electrophoresis in a 20% polyacrylamide gel in the presence of 7 M urea. The 0.6 $\times$ 10 cm cylindrical gel was fractionated into 1 mm slices that were dissolved in 18% H_2O_2 before counting in Aquasol (b). XC, xylene cyanol dye; BPB, bromophenol blue dye.

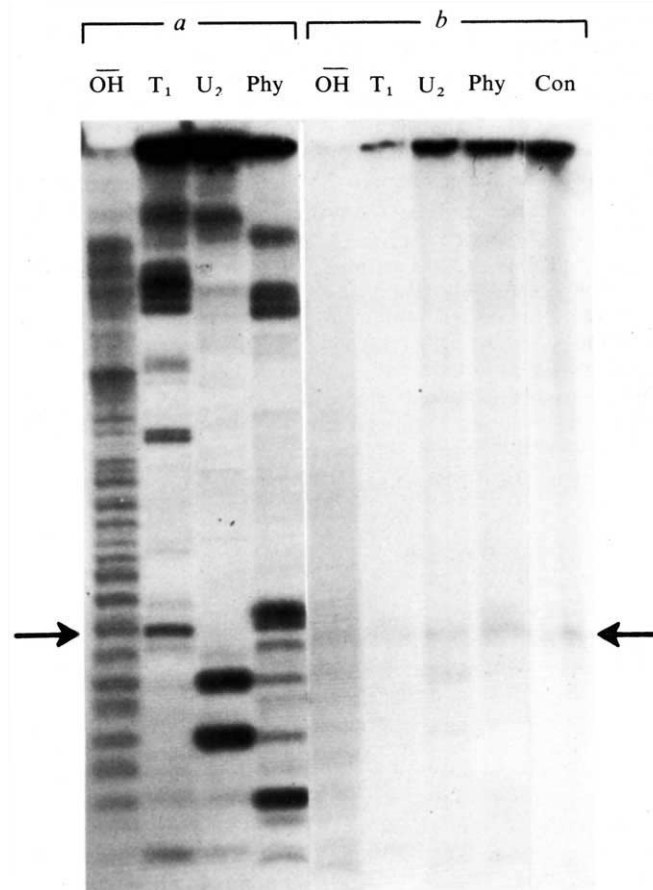


Fig. 5 Nucleotide sequence analyses of 18S rRNA and mRNA-rRNA complexes. AMT cross-linked complexes of mRNA and 18S rRNA that had been 3'-end labelled with ^{32}P -pCp. RNase T_1 digested and selectively bound to DBAE-cellulose two times (fractions 85–115 in Fig. 4a) were recovered by ethanol precipitation and irradiated with short wavelength UV light (254 nm) for 2 h at 0°C in conditions that photoreverse AMT cross-links³². After photoreversal, the ^{32}P -labelled fragments were analysed by partial enzymatic digestion and polyacrylamide gel electrophoresis³³ in comparison with 3'-terminally ^{32}P -labelled 18S rRNA. a, Wheat germ 18S rRNA labelled at the 3' end with ^{32}P -pCp; b, RNase T_1 fragment from photoreversed rRNA-mRNA complexes. Arrows indicate 18S rRNA 3'-terminal T_1 oligonucleotide. OH, alkaline hydrolysate; T_1 , U_2 and Phy, the respective RNase digests using conditions described previously⁴⁷; Con, RNase T_1 fragments from photoreversed complexes not further digested.

the double-labelled peak at fractions 46–47 in Fig. 4b. The formation of cross-links involving pyrimidine residues distal to the 3' end T_1 fragment of rRNA might have two related effects consistent with the higher $^{32}\text{P}/^3\text{H}$ ratio in the longer oligonucleotides: increased accessibility of the 3' end for ^{32}P -pCp addition by RNA ligase and decreased susceptibility for complete cleavage by RNase T_1 . Alternatively the radioactivity profiles may reflect cross-linking of some mRNA 5' ends to other sites in the 18S rRNA. Results similar to those in Fig. 4 were obtained with reovirus mRNA–18S rRNA cross-linked fragments from wheat 40S and rabbit 80S initiation complexes.

To confirm that the DBAE-bound, ^{32}P -labelled oligonucleotides included 3' ends of 18S rRNA, the cross-linked, double-labelled fragments eluted from the resin were irradiated at a wavelength of 254 nm to photoreverse the AMT cross-links³². The photoreversed sample was analysed in a polyacrylamide sequencing gel in comparison with 3'- ^{32}P -labelled wheat germ 18S rRNA³³. Partial enzymatic digests of the rRNA in non-denaturing conditions yielded patterns

consistent with the published 3' sequence⁵ including a nuclease-resistant, presumptive hairpin structure extending from about residue 10 to 30 (Fig. 5a). Although some of the radioactivity in the irradiated oligonucleotide sample remained near the gel origin, the released ³²P-labelled material migrated in a position corresponding to the T₁ limit oligonucleotide of rRNA (Fig. 5b, Con, arrows). This oligonucleotide yielded a series of shorter products on digestion with alkali (OH⁻). RNase T₁ treatment released some radioactivity which migrated near position 1 as in T₁ digests of rRNA, but most of the oligonucleotide remained undegraded (T₁). It was also relatively resistant to *Physarum* RNase I in these partial digestion conditions (Phy). RNase U₂ treatment yielded two additional bands that corresponded in position to oligonucleotides obtained from rRNA by U₂ digestion (U₂). These results indicate that the DBAE-selected, cross-linked fragments included some of the ³²P-pCp-labelled 3'-terminal T₁ oligonucleotide from 18S rRNA.

Role of base pairing

Several lines of evidence^{2,3,34-36} support the early suggestion that initiation codon selection in prokaryotic mRNAs is influenced by base pairing between the 3' sequence of 16S rRNA and a polypurine region about 10 nucleotides to the 5' side of the initiator triplet. Similar kinds of experiment in eukaryotes have not been reported, but sequences complementary to the conserved 3' end of 18S rRNA occur in the 5' noncoding regions of many eukaryotic mRNAs. In some cases the complementarity is striking, with calculated stabilities for the potential mRNA-rRNA base-paired structures in the same range as prokaryotic initiator regions^{15,37,38}, and in polyoma virus mRNA the 5' leader contains multiple copies of such a sequence³⁹. In other functional eukaryotic mRNAs, including species of reovirus³¹ and VSV mRNA⁴⁰, the potential for stable base pairing between 5' ends and 18S rRNA 3'-termini is so low as to suggest that it could have at most a minor influence on initiation, indicating that this type of base pairing is not obligatory for eukaryotic protein synthesis. The observation that the translation of prokaryotic virus mRNAs in wheat germ extract is strongly enhanced after enzymatic addition of a 5' cap supports this conclusion⁴¹.

In an effort to test experimentally whether mRNAs interact with rRNAs during eukaryotic initiation, we have used photochemical reaction with the RNA-RNA cross-linking agent, AMT. By using ³H-methyl-labelled capped mRNA and ³²P-pCp-labelling of rRNA, we detected cross-linking between mRNA 5' fragments and 18S rRNA 3' sequences in both 40S and 80S initiation complexes. It is not clear if cross-linking occurs at many sites in the 18S rRNA or is restricted to the 3' end. Although the findings indicate that the opposite ends of mRNA and 18S rRNA are proximally located at least in some initiation complexes, the interaction clearly is not strictly homologous to 'Shine and Dalgarno' base-paired structures. One obvious difference noted previously⁵ is the variation among eukaryotic mRNAs in the position of the rRNA complementary sequences relative to the initiator AUG, compared with the fixed position in prokaryotic mRNAs. However, since different 5' leaders precede the initiator codon in eukaryotic mRNAs and ribosomes apparently re-position at the 5'-proximal AUG after attaching near the cap⁶, 18S rRNA 3'-complementary sequences with functional effects on initiation could occur at different places within the 5' noncoding region of various mRNAs. Another consideration is that some mRNAs, and particularly synthetic ribopolymers such as capped poly(U), contain too few 5'-proximal nucleotides complementary to the 3' sequence of 18S rRNA to form a stable base-paired structure of the prokaryotic type. By the cross-linking method capped poly(U), as well as mRNAs like adenovirus hexon mRNA which can form a stable base-paired structure between 5' leader sequences and 18S rRNA 3' ends¹⁵, would be fixed to 18S rRNA by the formation of AMT

diadducts between pyrimidines that are even transiently juxtaposed. In some cases cross-linking presumably could also occur without base-pairing by the sequential reaction of AMT with two pyrimidines.

It is important to emphasize that although initiation-dependent interactions, perhaps base-pairing, between mRNA 5'-terminal regions and 18S rRNA can be detected by AMT cross-linking, their functional role if any in eukaryotic protein synthesis remains to be established. However, a stable interaction compatible with base-pairing has been demonstrated between 18S rRNA and globlin mRNA in 40S initiation complexes formed in a reconstituted mammalian cell-free protein synthesizing system (B. Erni and T. Staehelin, personal communication). It may be possible to test whether this interaction has a function in translation by using cloned rDNA including specific fragments complementary to the 3' end of 18S rRNA^{34,35,42}.

We thank Alba LaFiandra and Maureen Morgan for assistance, Drs M. Kozak and Y. Furuichi for discussion, Dr J. Hearst for ³H-AMT, and the following for gifts of virus or viral mRNA: Drs A. Marcus (TMV), J. Clark (STNV), Y. Furuichi (CPV), A. Banerjee (VSV) and R. Krug (influenza virus).

Note added in proof: Recent studies indicate that mRNA cross-linking is mainly within the ribosome-protected region.

Received 24 January; accepted 18 April 1980.

- Shine, J. & Dalgarno, L. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1342-1346 (1974).
- Steitz, J. A. & Jakes, K. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4734-4738 (1975).
- Dunn, J. J., Buzash-Pollert, E. & Studier, F. W. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2741-2745 (1978).
- Shine, J. & Dalgarno, L. *Nature* **254**, 34-38 (1975).
- Hagenbüchle, O., Santer, M., Steitz, J. A. & Mans, R. J. *Cell* **13**, 551-563 (1978).
- Kozak, M. *Cell* **15**, 1109-1123 (1978).
- Barelle, F. E. & Brownlee, G. G. *Nature* **274**, 84-87 (1978).
- Both, G. W. *FEBS Lett.* **101**, 220-224 (1979).
- De Wachter, R. *Nucleic Acids Res.* **7**, 2045-2054 (1979).
- Sprague, K. U., Steitz, J. A., Grenley, R. M. & Stocking, C. E. *Nature* **267**, 462-465 (1977).
- Schwartz, Z. & Kössel, H. *Nature* **279**, 520-522 (1979).
- de Jonge, P., Klootwijk, J. & Planta, R. J. *Nucleic Acids Res.* **4**, 3655-3663 (1977).
- Alberty, H., Raba, M. & Gross, H. J. *Nucleic Acids Res.* **5**, 425-434 (1978).
- Hunt, J. A. *Biochem. J.* **120**, 353-363 (1970).
- Zill, E. B. & Evans, R. M. *Cell* **15**, 1463-1475 (1978).
- Schroeder, Jr, H. W., Liarakos, C. D., Gupta, R. C., Randerath, K. & O'Malley, B. W. *Biochemistry* **18**, 5798-5808 (1979).
- Pathak, M. A., Krämer, D. M. & Fitzpatrick, T. B. in *Sunlight and Man: Normal and Abnormal Photochemical Responses* (eds Pathak, M. A. et al.) 335-387 (University of Tokyo Press, 1974).
- Isaacs, S. T., Shen, C. J., Hearst, J. E. & Rapoport, H. *Biochemistry* **16**, 1058-1064 (1977).
- Nakashima, K., LaFiandra, A. J. & Shatkin, A. J. *J. biol. Chem.* **254**, 8007-8014 (1979).
- Both, G. W., Furuichi, Y., Muthukrishnan, S. & Shatkin, A. J. *Cell* **6**, 185-195 (1975).
- Muthukrishnan, S., Morgan, M., Banerjee, A. K. & Shatkin, A. J. *Biochemistry* **15**, 5761-5768 (1976).
- Kozak, M. & Shatkin, A. J. *Meth. Enzym.* **60**, 360-375 (1979).
- Kozak, M. & Shatkin, A. J. *J. biol. Chem.* **251**, 4259-4266 (1976).
- Kozak, M. & Shatkin, A. J. *Cell* **13**, 201-212 (1978).
- Marcus, A. J. *J. biol. Chem.* **245**, 962-966 (1970).
- Trachsel, H., Erni, B., Schreier, M. H. & Staehelin, T. *J. molec. Biol.* **116**, 755-767 (1977).
- Both, G. W., Furuichi, Y., Muthukrishnan, S. & Shatkin, A. J. *J. molec. Biol.* **104**, 637-658 (1976).
- England, T. E. & Uhlenbeck, O. C. *Nature* **275**, 560-561 (1978).
- Rosenberg, M. *Nucleic Acids Res.* **1**, 653-671 (1974).
- Furuichi, Y., Shatkin, A. J., Stavnezer, E. & Bishop, J. M. *Nature* **257**, 618-620 (1975).
- Kozak, M. *Nature* **269**, 390-394 (1977).
- Rabin, D. & Crothers, D. M. *Nucleic Acids Res.* **7**, 689-703 (1979).
- Donis-Keller, H., Maxam, A. M. & Gilbert, W. *Nucleic Acids Res.* **4**, 2527-2538 (1977).
- Eckhardt, H. & Lüthmann, R. J. *J. biol. Chem.* **254**, 11185-11188 (1979).
- Taniguchi, T. & Weissmann, C. *Nature* **275**, 770-772 (1978).
- Roberts, T. M., Bikel, I., Yocum, R. R., Livingston, D. M. & Ptashne, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5596-5600 (1979).
- Azad, A. A. & Deacon, N. J. *Biochem. biophys. Res. Commun.* **86**, 568-576 (1979).
- Tsujiyama, Y. & Suzuki, Y. *Cell* **18**, 591-600 (1979).
- Legon, S. J. *J. molec. Biol.* **134**, 219-240 (1979).
- Rose, J. K. *Cell* **14**, 345-353 (1978).
- Rosenberg, M. & Paterson, B. M. *Nature* **279**, 696-701 (1979).
- Manning, R. F., Samols, D. R. & Gage, L. P. *Gene* **4**, 153-166 (1978).
- Furuichi, Y. & Shatkin, A. J. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3448-3452 (1976).
- Nakashima, K. & Shatkin, A. J. *J. biol. Chem.* **253**, 8680-8682 (1978).
- Nakashima, K., Chanda, P. K., Deutsch, V., Banerjee, A. K. & Shatkin, A. J. *J. Virol.* **32**, 838-844 (1979).
- Pelham, H. R. B. & Jackson, R. J. *Eur. J. Biochem.* **67**, 247-256 (1976).
- Darzynkiewicz, E. & Shatkin, A. J. *Nucleic Acids Res.* **8**, 337-350 (1980).

Monoclonal antibodies which recognize different cell types in the rat retina

Colin J. Barnstable

Department of Neurobiology, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115

Seven monoclonal antibodies have been produced against a membrane preparation from adult rat retina. Three antibodies reacted with particular regions of rat photoreceptor cell surfaces: RET-P1 labelled the cell bodies, outer and inner segments (rods but not cones), RET-P2 labelled only outer segments and RET-P3 labelled only the cell bodies. Three antibodies reacted with glial cells; RET-G2 and RET-G3 were specific for Müller cells, RET-G1 also labelled glia elsewhere in brain. The seventh antibody (RET-N1) reacted with many types of neuronal cells.

It is widely believed that differences between classes of neural cells are expressed in part by distinct patterns of cell-surface molecules¹⁻³. Cell-surface molecules such as transmitter receptors and ion channels are basic to the function of the nervous system^{4,5}. These and other surface molecules may be important in determining the exquisite specificity of synaptic connections.

Antibodies are almost uniquely suited for the detection of such cell-surface molecules since they can be used in histochemical assays to resolve single cells in mixed cell populations; they can be used in quantitative biochemical assays to determine the amount, molecular nature and tissue distribution of the antigens recognized; and perhaps most importantly, they can be raised against essentially any antigen or cell type of interest. Progress in producing antibodies against any but the broadest classes of neural cell types has, until recently, been hindered by the heterogeneity of the immune response generated against the available antigens⁶⁻⁹. The technique introduced by Milstein and co-workers^{10,11}, whereby antibodies are produced by cloned cell lines, has overcome many of these difficulties. Such monoclonal antibodies have already been of great value in biochemical, functional and genetic analyses of cell surface molecules¹²⁻¹⁵.

We have chosen the visual system, and particularly the retina, as a model system in which to study the differences in cell-surface molecules of different subclasses of neural cells. This system has the advantage that many of the cells involved in the transmission of visual information from the photoreceptors to the primary visual cortex have been identified and reasonably well characterized with respect to their physiological responses and synaptic interactions¹⁶⁻¹⁸. The retina has the additional advantages that it is readily accessible, it has only seven basic neural cell types, its layered structure generally allows assignment of cell type from position alone¹⁹, and it is a region of the vertebrate central nervous system (CNS) from which single dissociated cells have been isolated and successfully used in physiological²⁰ and biochemical studies^{21,22}.

This article describes initial observations on seven monoclonal antibodies that recognize cell-surface molecules of rat retina. Three of these antibodies react with photoreceptors, although each stains subregions of the cell surface in a distinct way. Three antibodies react with Müller cells, the main glial cell type of retina, and the seventh reacts with many classes of neuronal cells throughout the brain.

protein in Freund's adjuvant 3 weeks apart. Three weeks after the second injection mice were given an intravenous injection of 100 µg of membrane protein in saline. Four days later spleens were removed and used for fusions. Spleen cells were fused with either P3-NS1/1-Ag4-1 or 8653 myeloma cells (gifts of C. Milstein and P. Goodfellow, respectively), and hybrid cultures selected and cloned as described previously^{15,24}.

Hybrid- and clone-culture supernatants were tested for antibody activity using an indirect trace radioactive binding assay as described by Williams²⁵. Target particles consisted of adult rat retina that had been lightly homogenized and fixed with 0.5% paraformaldehyde²⁶. Bound mouse immunoglobulin was detected using a ¹²⁵I-labelled F(ab')₂ fragment of an affinity-purified rabbit anti-mouse IgG antibody.

The seven antibodies described here have been selected from the products of four separate fusions. All seven antibodies were found to be of the IgG1 subclass when tested by Ouchterlony double diffusion against subclass-specific sera.

The antibodies were initially selected for study because the early culture supernatants did not react in a binding assay with rat thymocytes or by immunofluorescence with a fibroblast cell line grown out from newborn rat lung (data not shown). Thus they seemed to recognize antigens that were not expressed by all rat tissues. To study the antigen expression by different tissues in a more quantitative way, each antibody was absorbed with crude membrane preparations from retina, cerebral cortex and cerebellum, with liver as a control non-neural tissue. The amount of membrane needed to inhibit antibody-binding activity by 50% was determined (see Table 1 legend) and was used as a measure of the level of antigen expression of that particular tissue (Table 1). Because these

Table 1 Tissue distribution of antigens recognized by anti-retina antibodies

Antibody	Antigenic activity of membrane			
	Retina	Cortex	Cerebellum	Liver
RET-P1	50	<1	<1	<1
RET-P2	2,326	<1	<1	<1
RET-P3	15	<1	<1	<1
RET-G1	238	1,724	600	<1
RET-G2	87	<1	1.3	7
RET-G3	100	<1	5	8
RET-N1	312	91	161	<1

Crude membrane fractions²³ were prepared from each tissue. Aliquots of monoclonal antibody solution, at a dilution chosen so as to be limiting in the assay, were incubated with varying concentrations of membranes for 2 h at 4°C. The residual antibody activity was then measured using a trace radioactive binding assay. At least 10 dilutions of each membrane preparation were used with each antibody. The antigenic activity is defined as the dilution of a 20 mg membrane protein per ml solution necessary to give 50% inhibition of the binding activity of an equal volume of antibody solution. The values given in the table are taken from inhibition curves, the points of which were each the mean of two separate inhibitions each assayed in duplicate.

Production and immunological characterization of antibodies

Retinas were dissociated from adult CD rats (Charles River Breeding Laboratories) and a crude membrane pellet prepared as described by Barclay *et al.*²³. Female BALB/c mice were given two intraperitoneal injections of 200 µg of membrane

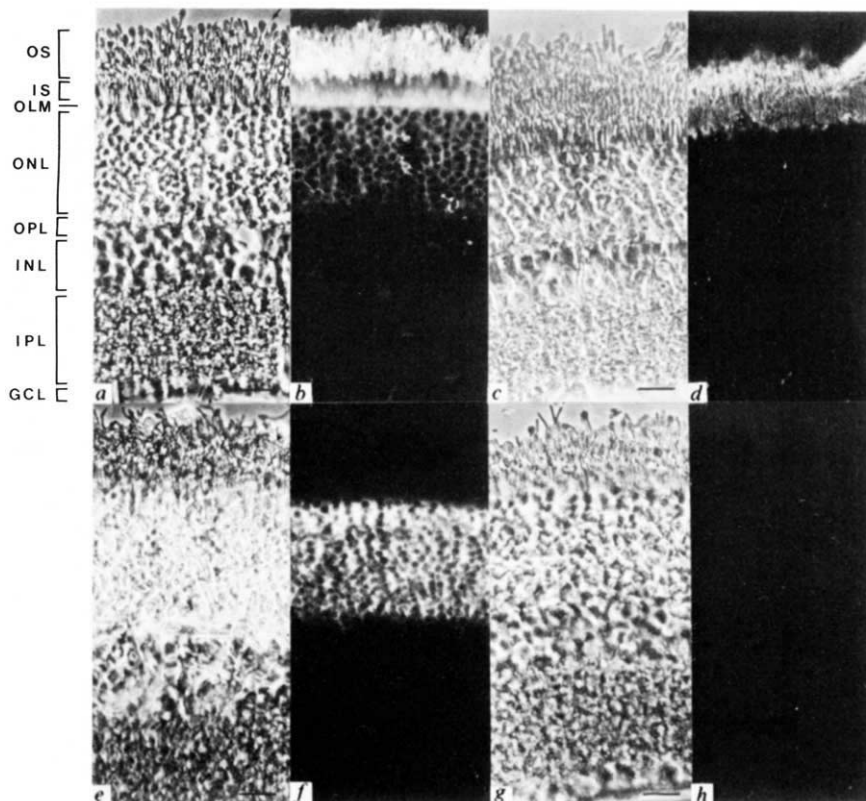


Fig. 1 Immunofluorescent labelling of sections of adult rat retina by antibodies RET-P1, RET-P2 and RET-P3. Rats were killed by cervical dislocation and the retinas rapidly dissected. They were fixed for 1 h at 4°C in 1% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.6. After fixation the retinas were soaked in 30% (w/v) sucrose overnight. Sections (15 µm) were cut on a freezing microtome and dried onto subbed slides. Sections were preincubated with 5% (w/v) bovine serum albumin (BSA) in phosphate buffer for 1 h at 4°C and then with the primary antibody for 18 h at 4°C. The slides were washed in two changes of a large excess of phosphate buffer for 10 min each at 4°C. Sections were then treated with a rhodamine-conjugated goat anti-mouse IgG preparation (Cappel) which was diluted in a solution of equal parts of 5% BSA in phosphate buffer and normal rabbit serum. Before use, the rabbit serum was passed over a column of mouse IgG coupled to Sepharose 4B to remove any heterophile anti-mouse IgG antibodies. After incubation for 45 min at room temperature the slides were again washed with two changes of phosphate buffer and then a coverslip applied using 50% glycerol in phosphate buffer as mounting medium. Slides were examined with a Zeiss ICM35 microscope. White light photographs were taken with phase contrast optics. Fluorescence photographs were taken using epi-illumination and excitation-barrier filter and reflector combination 48-77 15-9902. Photographs were taken on H & W Control film with 2 min exposures for fluorescence and an automatic light meter for phase contrast photographs. *a, b*, RET-P1 antibody; *c, d*, RET-P2 antibody; *e, f*, RET-P3 antibody; *g, h*, control using a monoclonal antibody against a human cell-surface glycoprotein. *a, c, e, g*, Phase contrast; *b, d, f, h*, fluorescence. The arrow at the top right corner of *a* indicates the only outer segment that can be detected as unlabelled in *b*. OS, outer segments; IS, inner segments; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar represents 20 µm.

values are functions of both quantity of antigen and antibody affinity, only comparisons between different tissues for the same antibody are meaningful. From Table 1 it can be seen that RET-P1, RET-P2 and RET-P3 antigens were predominantly if not exclusively localized in retina because only retina membrane was able to give measurable inhibition of antibody binding activity. RET-G2 and RET-G3 antigens were expressed strongly by retina, weakly by cerebellum and liver and not detectably by cortex. Neither antigen was detectable by immunofluorescent staining of cerebellum or liver sections (data not shown) so that it is not yet possible to ascribe the absorption to the presence of a few cells with high levels of antigen expression, many cells with low levels of antigen expression or even some nonspecific absorption that affects these two antibodies but not the others. RET-G1 and RET-N1 antigens were strongly expressed by all three brain regions but were not detectable on liver. Although the absorption has the advantage of being quantitative, it cannot be used to determine which cell types in a tissue are expressing the antigen. For this, immunofluorescent staining of tissue sections was carried out.

Antibodies RET-P1, RET-P2 and RET-P3 specifically label photoreceptors

The immunofluorescent staining of sections of adult rat retina given by RET-P1 is shown in Fig. 1*a* and *b*. With the exception of the outer plexiform layer, the whole of the photoreceptor layer was labelled. Clear ring fluorescence was found around almost all of the photoreceptor cell bodies. The packing between the cell bodies appeared looser towards the outer limiting membrane and the intervening spaces often showed fluorescence. This was probably due to the fibres which connect the cell bodies with their inner segments²⁷. The

photoreceptor inner segments were labelled at about the same intensity as the cell bodies but the outer segments had a much brighter fluorescence. These differences presumably represented differences in antigen density which supports previous observations that the composition of the outer segment membrane is different from that of the rest of the cell^{28,29}. This was much more decisively demonstrated by the staining pattern given by antibodies RET-P2 (Fig. 1*c, d*) and RET-P3 (Fig. 1*e, f*). Within the limitations of the fluorescence assay, RET-P2 antigen was located exclusively on the outer segments and RET-P3 antigen was located exclusively on the photoreceptor cell bodies. These differences in labelling patterns seen with these three antibodies suggest that some photoreceptor membrane molecules are common to all regions of the cell while others are restricted to discrete areas of the cell. It is not yet known whether this restriction is caused by some form of anchorage of particular molecules in a fluid membrane to submembranous or extracellular elements or by localized areas of insertion of newly synthesized molecules in a non-fluid membrane.

Experiments are now under way to determine the chemical nature of the RET-P1, RET-P2 and RET-P3 antigens to see whether both membrane proteins and lipids are restricted in their localization. In addition, production of other antibodies that label specific regions of photoreceptors will allow an estimate of the number of compartments into which the photoreceptor cell membrane is divided.

Although RET-P2 antibody was not absorbed by cerebellum membrane, immunofluorescent labelling of cerebellum sections showed that it reacted with structures at the level of the initial segment of the Purkinje cell (data not shown). The exact nature of the labelled structures and their relationship to the photoreceptor outer segments have yet to be determined. Neither RET-P1 nor RET-P3 stained any cerebellar structures.

The rat retina consists almost entirely of rods³⁰, so that judging whether or not the three antibodies reacted with both rods and cones was almost impossible. To investigate this, the three antibodies were tested on a number of different species to see whether or not any of them reacted with a retina containing a higher proportion of cones. RET-P2 and RET-P3 seem to be specific for rat but RET-P1 reacted with several species (including BALB/c mice from which the antibody-producing spleen cell was derived). A group of photoreceptors in a section of retina from the tiger salamander (*Ambystoma tigrinum*) is shown in Fig. 2. Cells 1, 3 and 5 are rods although the outer segment of cell 3 was removed during dissection. All three cells stain throughout their length with the most intense fluorescence being in the outer segment. The bright band at the junction of the inner and outer segments was probably due to labelling of the deep membrane invaginations at this region. These invaginations have been shown to represent the initial stage of pinching off of the disks of the outer segment³¹. Cells 2 and 4 have the morphology of cones, that is, narrower cell bodies, narrower but longer inner segments and slender outer segments. The outer segments of cells 2 and 4 are almost certainly damaged but there is no trace of fluorescence on any part of the cell. This would indicate that antigen RET-P1 is restricted to rods. As can also be seen in Fig. 2, the axons and synaptic pedicles of the rods were labelled. In several cases fine labelled processes were found to be given off from the thick pedicles. The lack of staining of the outer plexiform layer in the rat retina found with this antibody may simply be because such staining was below the threshold for visualization or alternatively may be real and may represent a difference in antigen compartmentalization between the two species.

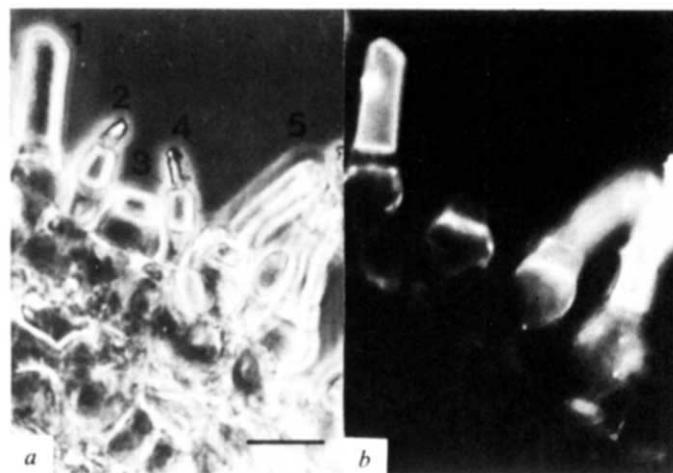


Fig. 2 Immunofluorescent labelling of tiger salamander photoreceptors by antibody RET-P1. Sections were prepared and treated as described in Fig. 1 legend except that the animals were dark-adapted for 4 h before being killed. *a*, Phase contrast; *b*, fluorescence. Scale bar represents 20 μ .

One obvious candidate for a photoreceptor-specific marker is the light-sensitive molecule rhodopsin. A rabbit antiserum raised against bovine rhodopsin stained both the outer and inner segments of frog photoreceptors³². This pattern was not given by any of the three antibodies described here. In addition, the anti-rhodopsin serum stained both rods and cones. None of these antibodies was inhibited by a partially purified preparation of bovine rhodopsin (a gift of Dr E. A. Schwartz) used at concentrations where whole retina membrane gave complete inhibition (data not shown). Thus, it is unlikely that any of these three antibodies recognize rhodopsin but final resolution of this must await a chemical analysis of the respective antigens.

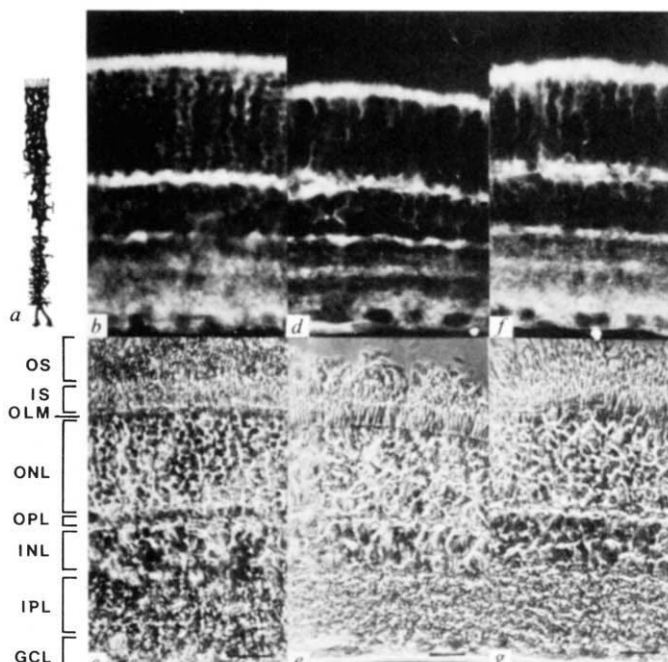


Fig. 3 Immunofluorescent labelling of sections of adult rat retina by antibodies RET-G1, RET-G2 and RET-G3. Sections were prepared and treated as described in Fig. 1 legend. *a*, A Müller cell taken from Golgi-stained retina as drawn by S. R. Cajal (ref. 35, p. 298); *b*, *c*, RET-G1 antibody; *d*, *e*, RET-G2 antibody; *f*, *g*, RET-G3 antibody. *b*, *d*, *f*, Fluorescence; *c*, *e*, *g*, phase contrast. Designation of retinal layers is described in Fig. 1 legend. Scale bar represents 20 μ .

Antibodies RET-G1, RET-G2 and RET-G3 specifically label Müller cells

Müller cells have a unique morphology in that they are the only cell type to extend radially across the retina from the outer limiting membrane to the inner limiting membrane^{33,34}. The essential features of their structure, as shown in the Golgi-stained cell (Fig. 3*a*), were clearly picked out in the immunofluorescent labelling of sections of adult rat retina given by antibodies RET-G1 (Fig. 3*b*, *c*), RET-G2 (Fig. 3*d*, *e*) and RET-G3 (Fig. 3*f*, *g*). At the outer limiting membrane, each antibody labelled the fine finger-like Müller cell processes that extend up between the inner half of the photoreceptor inner segments. Thick radial processes passing through the outer nuclear layer were also labelled although the single focal plane shown in the photographs was not always adequate to show a single process traversing the whole width of that layer. Such fibres could frequently be followed visually. Virtually no tangential labelling was seen in the outer nuclear layer, indicating that the photoreceptor cell bodies had not reacted with the antibodies. The labelling in the inner nuclear layer was harder to interpret but probably represented Müller fibres following the contours of the cell bodies in this region on their way to the inner plexiform layer.

In very few cases was it possible to see a clear ring of fluorescence around a cell body as would have been expected if the neuronal cell bodies had been labelled. Those few cell bodies that were labelled had the position expected for Müller cell bodies. The radial Müller fibres give off fine tangential processes in the plexiform layers causing both plexiform layers to be labelled. The labelling of the inner plexiform layer showed three bands of higher intensity. One band was located at the junction of the inner nuclear and inner plexiform layers, one band was about half way through the inner plexiform layer and the other thick band was at the junction of the inner plexiform and ganglion cell layers and was continuous with the labelling of the bulbous feet that surround the ganglion cell bodies and optic fibres and end at the inner limiting membrane. This banded appearance of the inner plexiform

layer, which probably reflects the density of tangential glial projections, was closely similar to the autoradiographic staining of Müller cells seen following uptake of radioactive GABA *in vitro* (refs 36, 37 and unpublished observations with R. W. Baughman).

As a further demonstration that antibody RET-G1 was reacting with Müller cells, immunofluorescent labelling of enzymatically dissociated adult rat retina cells³⁸ was carried out. The results (Fig. 4) clearly show that this antibody labelled cells with the morphology characteristic of Müller cells. Cells with the morphology of bipolar cells and a variety of amacrine cells were not labelled in these preparations.

Müller cells and astrocytes have a number of structural and functional features in common, such as close apposition to neuronal processes³⁹, potassium ion transport⁴⁰, and γ -aminobutyric acid transport⁴¹. Thus it was not surprising to find an antibody, RET-G1, that reacted with glial cells in all regions of brain. The results in Table 1 show that cerebral cortex and cerebellum express more RET-G1 per mg membrane protein than retina although the differences provide no information about the relative amounts expressed per cell.

In cerebellum, the cells expressing this antigen were found in all layers. The area illustrated in Fig. 5c shows labelled cell bodies in the granular layer and processes passing up through the unlabelled Purkinje cell layer to the molecular layer. Further studies will be necessary to determine whether this antigen is also on oligodendrocytes and glia of the peripheral nervous system. RET-G2 and RET-G3 antigens were expressed by Müller cells but not to a significant extent by other glia. The nature and function of these two 'Müller-cell specific' antigens are unknown. The two can be distinguished by their fluorescent labelling pattern on adult retina. RET-G2 antibody gave a relatively even labelling but RET-G3 antibody gave a highly punctate pattern of labelling in the inner plexiform layer. Studies at the electron microscope level will show whether these areas of increased labelling are related in any way to other structures in the inner plexiform layer, such as areas of glial processes close to synapses.

Antibody RET-N1 recognizes a neurone-specific antigen

Antibody RET-N1 labelled several cell types in sections of adult rat retina (Fig. 5a). It labelled the whole length of the photoreceptor inner segments but not the outer segments or cell bodies although some very faint labelling was found over

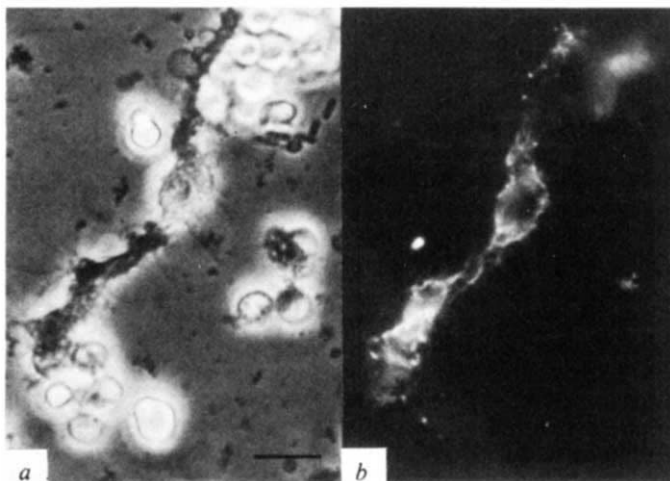


Fig. 4 Immunofluorescent labelling of enzymatically dissociated Müller cells by RET-G1 antibody. Retinas were dissected from adult rats and cells dissociated using published procedures³⁸. The cell suspension was allowed to settle to the bottom of Petri dishes and the labelling carried out using 1-h incubations at room temperature for primary and fluorescent antibodies. Three rinses with HEPES-buffered Hank's salts solutions were carried out after each antibody incubation, care being taken not to disturb the cells. Scale bar represents 10 μ .

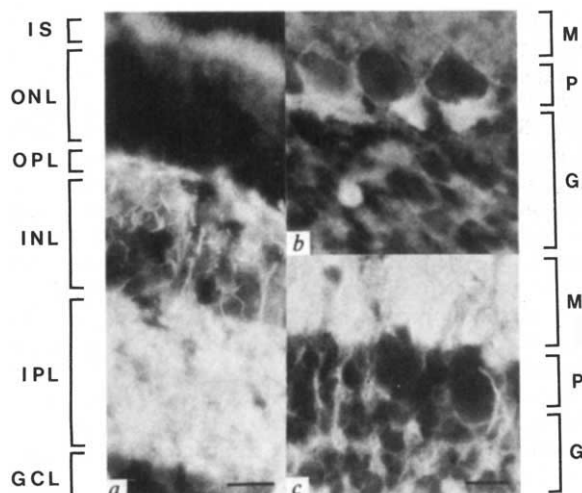


Fig. 5 *a*, Immunofluorescent labelling of adult rat retina by antibody RET-N1. The section preparation, labelling and the designation of retinal layers is as in the legend to Fig. 1. *b*, Immunofluorescent labelling of adult rat cerebellum by antibody RET-N1. *c*, Immunofluorescent labelling of adult rat cerebellum by antibody RET-G1. For *b* and *c* animals were anaesthetized with Nembutal and perfused through the ventricle with 2% paraformaldehyde in phosphate buffer. Cerebella were then dissected and treated as for retina. M, molecular layer; P, Purkinje cell layer; G, granular layer. Scale bar represents 20 μ .

the outer nuclear layer. Both plexiform layers were stained and many cell bodies in the outer half of the inner nuclear layer could be recognized. Axons of bipolar cells could be seen leaving some of these cells and passing through to the inner plexiform layer. There seemed to be fewer labelled cells in the inner half of the inner nuclear layer which may indicate that some amacrine cells were unlabelled. The ganglion cells were labelled. No traces of the characteristic glial labelling pattern—radial fibres in the outer nuclear layer and glial feet around the ganglion cells—were found. The absorption results (Table 1) indicated that RET-N1 antibody reacted with cortex and cerebellum. In agreement with this, the antibody was found to label cells at all levels in the cerebral cortex (data not shown). In cerebellum it also labelled several cell types. In the portion shown (Fig. 5b) it labelled Purkinje cells and brightly labelled pinceaux of basket cell terminals around the Purkinje cell initial segment. Many other cell bodies in the granular layer and processes in the molecular layer were also labelled, although in a pattern different from that given by antibody RET-G1. Thus antigen RET-N1 may be a marker for most if not all neurones of the CNS although further work will be necessary to show whether or not it cross-reacts with types of glial cells not found in the retina such as oligodendrocytes. It will also be of interest to determine whether antibodies RET-G1 and RET-N1 label glia and neurones, respectively, of the peripheral nervous system as these are of neural crest rather than neural tube embryonic origin. Experiments to investigate this are under way.

Discussion

The results presented above show clearly that monoclonal antibodies can be produced against subclasses of neural cells. It has yet to be shown formally that each antibody interacts with an integral cell-surface molecule rather than extracellular matrix or intracellular membrane material, but this seems likely. Fixation by itself is not an efficient way of allowing visualization of internal antigens in tissue sections. Addition of detergent to sections similar to those illustrated neither changed nor enhanced the labelling pattern, suggesting that external rather than internal antigens were recognized. Final demonstration of the cell-surface location of the antigens will be achieved by a combination of electron microscopic immunocytochemistry, biochemistry and studies in tissue culture on living cells.

The antibodies described here will be valuable for several studies. For example, they will be useful for studying the topography and development of particular cell types in the intact organism. Preliminary experiments have established that RET-P1, RET-P2 and RET-P3 are expressed at different stages of photoreceptor development and RET-G1, RET-G2 and RET-G3 are expressed at different stages of Müller cell development. Thus they are markers not only for adult cells but also for immature cells at discrete stages of development. Whether such stages are intrinsic to neural cells after the initial step of determination or whether they require distinct environmental triggers is not known. Experiments are in progress to test these alternatives by following cell maturation in defined *in vitro* culture systems.

Another use of antibodies such as those described above, and others now being characterized, will be for the separation of cells and subcellular fractions from adult and developing

tissue for a variety of physiological and biochemical studies.

There is also the additional hope that production of antibodies that recognize particular neural cell subclasses may lead to the identification of cell-surface molecules that mediate functions specific to those subclasses and may, therefore, aid in an understanding of the molecular and genetic basis of neural cell development and function.

I thank Torsten Wiesel for his continuing encouragement and support of this work, Peter MacLeish for help with the salamander retina, Robert Baughman, Ed Furshpan, Peter Parham and Elio Raviola for their comments on the manuscript, Lynn Mangini, Karl Reich and Jane Soukup for technical assistance, and Cathy Cross for photography. This work was supported by grant EY00606 from the National Eye Institute, NIH, and fellowship DRG-242 from the Damon Runyon-Walter Winchell Cancer Fund.

Received 12 February; accepted 17 April 1980.

1. Sidman, R. L. *3rd Lepetit Colloquium on Cell Interactions* (ed. Silvestri, L. G.), 1-13 (North-Holland, Amsterdam, 1972).
2. Moscona, A. A. (ed.) *The Cell Surface in Development* (Wiley, New York, 1974).
3. Rutishauser, V., Brackenbury, R., Thiery, J. P. & Edelman, G. M. *Birth Defects: Original Article Series XIV*, 305-316 (1978).
4. Krnjevic, K. *Physiol. Rev.* **54**, 418-540 (1974).
5. Hall, Z., Kelly, R. & Fox, C. F. (eds) *Cellular Neurobiology* (Liss, New York, 1977).
6. Fields, K. L., Gosling, C., Megson, M. & Stern, P. L. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1296-1300 (1975).
7. Schachner, M., Ruberg, M. Z. & Carnow, T. B. *Brain Res. Bull.* **1**, 367-377 (1976).
8. Stallcup, W. B. & Cohn, M. *Expl. Cell Res.* **98**, 285-297 (1976).
9. Raff, M. C. *et al. Brain Res.* **174**, 283-308 (1979).
10. Köhler, G. & Milstein, C. *Nature* **256**, 495-497 (1975).
11. Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W. & Howard, J. C. *Nature* **266**, 550-552 (1977).
12. Standing, R., McMaster, W. R., Sunderland, C. A. & Williams, A. F. *Eur. J. Immun.* **8**, 832-839 (1978).
13. White, R. A. H. *et al. J. exp. Med.* **14**, 664-673 (1978).
14. Reinherz, E. L., Kung, P. C., Goldstein, G. & Schlossman, S. F. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4061-4065 (1979).
15. Barnstable, C. J. *et al. Cell* **14**, 9-20 (1978).
16. Werblin, F. S. & Dowling, J. E. *J. Neurophysiol.* **32**, 339-355 (1969).
17. Hubel, D. H. & Wiesel, T. N. *J. Physiol., Lond.* **155**, 385-398 (1961).
18. Hubel, D. H. & Wiesel, T. N. *Proc. R. Soc. B* **198**, 1-59 (1977).
19. Dowling, J. E. & Boycott, B. B. *Proc. R. Soc. B* **166**, 80-111 (1966).

20. Bader, C. R., MacLeish, P. R. & Schwartz, E. A. *J. Physiol., Lond.* **296**, 1-26 (1979).
21. Lam, D. M. K. *Cold Spring Harb. Symp. quant. Biol.* **40**, 571-579 (1975).
22. Sarthy, V. P. & Lam, D. M. K. *J. Neurochem.* **32**, 455-461 (1979).
23. Barclay, A. N., Letarte-Muirhead, M. & Williams, A. F. *Biochem. J.* **151**, 699-706 (1975).
24. Brodsky, F. M., Parham, P., Barnstable, C. J., Crumpton, M. J. & Bodmer, W. F. *Immun. Rev.* **47**, 3-61 (1979).
25. Williams, A. F. *Contemporary Topics molec. Immun.* **6**, 93-116 (1977).
26. Barclay, A. N. *Brain Res.* **133**, 139-143 (1977).
27. Cajal, S. R. *The Structure of the Retina* (Translators Thorpe, S. A. & Glickstein, M.) (Thomas, Illinois, 1972).
28. Young, R. W. *J. Ultrastruct. Res.* **23**, 462-473 (1968).
29. Papermaster, D. S., Converse, C. A. & Zorn, M. A. *Expl. Eye Res.* **23**, 105-116 (1978).
30. La Vail, M. M. *Invest. Ophthalm.* **15**, 64-70 (1976).
31. Cohen, A. I. *Vision Res.* **10**, 445-453 (1970).
32. Dewey, M. M., David, P. K., Blasie, J. K. & Barr, L. J. *molec. Biol.* **39**, 395-405 (1969).
33. Uga, S. & Smelser, G. K. *Invest. Ophthalm.* **12**, 295-307 (1973).
34. Miller, R. E. & Dowling, J. E. *J. Neurophysiol.* **33**, 323-341 (1970).
35. Cajal, S. R. *Histologie du Systeme Nerveux de l'Homme et des Vertebres Vol. II* (CSIC, Madrid, 1955).
36. Neal, M. J. & Iversen, L. L. *Nature new Biol.* **235**, 217-218 (1972).
37. Voaden, M. J. in *Transmitters in the Visual Process* (ed. Bonting, S. L.) 107-125 (Pergamon, Oxford, 1976).
38. Sarthy, P. V. & Lam, D. M. K. *Brain Res.* **176**, 208-212 (1980).
39. Peters, A., Palay, S. L. & Webster, H. de F. *The Fine Structure of the Nervous System* (Saunders, Philadelphia, 1976).
40. Kuffler, S. W., Nicholls, J. G. & Orkand, R. K. *J. Neurophysiol.* **36**, 855-868 (1966).
41. Iversen, L. L. & Kelly, J. S. *Biochem. Pharmac.* **24**, 933-938 (1975).

LETTERS

Pressure balance and pressure distribution along the dayside ionopause of Venus

O. L. Vaisberg*, D. S. Intriligator† & V. N. Smirnov*

* Space Research Institute, USSR Academy of Sciences, Moscow, USSR

† University of Southern California, Los Angeles, California 90007

The pressure distribution within the magnetic barrier just outside the ionopause of Venus is shown here to be similar to expected solar wind pressure variation provided the more realistic shape of the ionopause is taken into account. The ionospheric pressure just below the magnetic barrier is about equal to the solar wind pressure while magnetic field pressure within the magnetic barrier amounts to about two-thirds of both ionospheric and solar wind pressures. So we can expect that one-third of magnetic barrier pressure is accounted for by the hot plasma contribution.

The existence of a bow shock near Venus was established during the Mariner 5 fly-by mission¹ and Venera 4 lander mission² in 1968. The close location of this shock to the planet demonstrated that Venus does not have a significant internal magnetic field and that the ionosphere of the planet is capable of deflecting a major part of the incoming solar wind plasma.

The Venera 9 and Venera 10 mission in 1975-76 made detailed studies of the shock and the wake region of Venus³⁻⁵. Recently the Pioneer Venus Orbiter (PVO) performed important measurements of the structure of the venusian ionosphere and its interaction with the solar wind^{6,7}. Above 200 km the dayside ionosphere is in a state of diffusive equilibrium with the O⁺ ions as the main constituent. The elevated electron and ion temperatures in the ionosphere together with the number density below the ionopause provide, in most cases, enough pressure to sustain the solar wind dynamic pressure. The height of the ionopause is strongly influenced by the solar wind pressure⁸⁻¹².

The magnetic field measurements from the PVO show that the magnetic pressure within the ionosphere is in most cases negligible, but that above the ionopause there is a region of increased magnetic field where the magnetic field pressure approximately equals the solar wind dynamic pressure. This region of increased magnetic field presumably transfers solar wind pressure to the ionosphere^{10,13-16}.

Joint data on the solar wind, magnetic field and ionosphere are available only for the first period of PVO observations in December 1978 when the region of solar zenith angle (SZA) $\geq 61.5^\circ$ was studied. It is frequently assumed that external pressure exerted on the venusian ionopause changes as a simple newtonian law of specular reflection, $\cos^2 \psi$ (where ψ is the SZA). But the deviations of the ionopause shape from that of a spherical surface should give rise to significant deviations from the $\cos^2$ law for large SZA. Further improvement may be

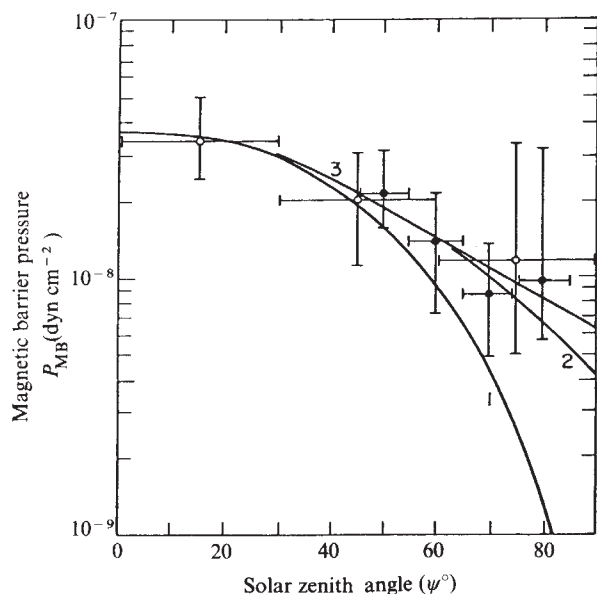


Fig. 1 Dependence of magnetic field pressure in the magnetic barrier on solar zenith angle. ●, From ref. 19; ○, from ref. 16. Curves are: 1, $\cos^2 \psi$; 2, $\cos^2 \chi$, with χ equal to expected angle between normal to ionopause and solar wind flow direction; and 3, hydrodynamic pressure variation along the boundary^{17,18}. Curves are normalized to subsolar pressure value $3.7 \times 10^{-8} \text{ dyn cm}^{-2}$.

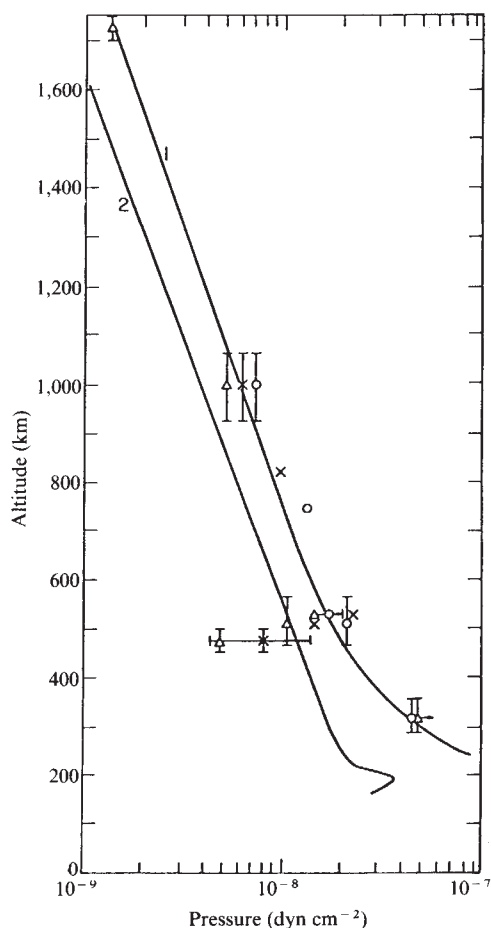


Fig. 2 Solar wind pressure (P_{SW}), magnetic barrier pressure (P_{MB}), increased by a factor of 1.5, and total (kinetic and magnetic) pressure of the ionosphere (P_{T}) below the ionopause plotted against observed ionopause altitudes on first orbits of PVO. Line 1 is drawn to show the change of pressure values at the ionopause with altitude for SZA range 65° – 75° . Mean ionospheric pressure model, line 2, obtained from means of measured number densities and ion and electron temperatures for the same SZA range^{6,7}. ○, P_{SW} ; △, P_{T} ; ×, $1.5 \times P_{\text{MB}}$.

obtained by taking into account a more realistic approximation of the external pressure. To obtain an estimate of the external pressure variation with SZA we assumed that the surface of the ionopause can be obtained from a simple ionospheric pressure balance^{17,18}. The parameter H/r_0 , ratio of the ionospheric scale height to planetocentric subsolar dimension of obstacle, defining the approximate shape of the ionopause, was taken equal to 0.07 (ref. 10). Figure 1 shows that the mean magnetic field pressure in the region just outside the ionopause, or in the 'magnetic barrier' closely follows the expected pressure variation along the boundary of the obstacle. The mean magnetic pressure within the magnetic barrier and its variations were taken from refs 16, 19. To fit the observations the pressure curves were normalized to $3.7 \times 10^{-8} \text{ dyn cm}^{-2}$ at $\psi = 0$ and this value is representative for mean subsolar magnetic barrier pressure for the PVO period of observations. Data of Fig. 1 confirm that pressure within the magnetic barrier is determined by the pressure of external flow and show that this condition holds up to SZA $\sim 90^\circ$.

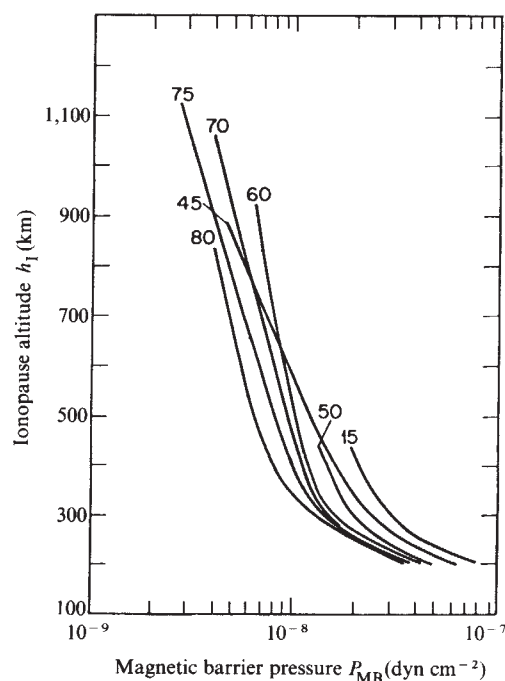


Fig. 3 Magnetic barrier pressure distribution versus height for different solar zenith angles (indicated) obtained according to data of refs 16, 19.

Distribution of measured values of ionospheric pressure just below the ionopause, magnetic pressure within the magnetic barrier, and solar wind pressure versus height of the ionopause for December 1978 are shown in Fig. 2. Data are limited to SZA between 63° and 90° . Solar wind pressure was estimated from the measured ρV^2 value corrected for pressure distribution along the boundary (curve 3, Fig. 1). Magnetic field pressure was multiplied by a factor of 1.5 to fit both the solar wind pressure and ionospheric pressure. Figure 2 shows good agreement between the measured ionospheric pressure below the ionopause and the solar wind pressure corrected for angle of incidence. It shows also that although the magnetic barrier has a major role in the transfer of solar wind pressure to the ionosphere, the plasma pressure within the magnetic barrier may provide up to one-third of the total pressure.

Comparison of the behaviour of the ionospheric pressure just below the ionopause with the mean ionospheric pressure distribution for the same SZA range apparently shows significant differences between these distributions suggesting modification of the subionopause layer due to changing solar wind conditions.

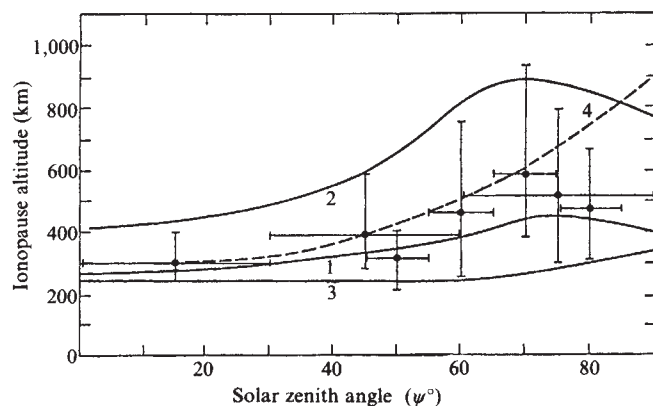


Fig. 4 Observed ionopause altitudes versus solar zenith angle. Expected 'instant' ionopause profiles are shown for close to the mean solar wind ram pressure value (curve 1); one half of it (curve 2); and doubled mean pressure value (curve 3). Dashed line, model ionopause profile for $H/r_0 = 0.07$ and $r_0 = 6,350$ km.

The magnetic pressure distribution within the magnetic barrier as a function of the ionopause altitude and as a function of SZA was obtained using data from (refs 16, 19). This distribution, shown in Fig. 3 enables the 'instant' profile of the ionopause to be found for a given solar wind pressure, provided that the hot plasma contribution to the magnetic barrier pressure is taken into account. Assuming that the plasma pressure within the magnetic barrier is one-third everywhere, the shapes of the ionopause for close to the mean solar wind condition, half and double this value were obtained. Figure 4 shows that the behaviour of the model profiles is in reasonable agreement with the observed mean ionopause altitude dependency on SZA and with the observed distribution of ionopause crossings. Also, it can be seen that both the observational data and the ionopause model show that at SZA $\sim 70^\circ$ the shape of the venusian ionopause differs significantly from the simple hydrostatic equilibrium model of the ionopause (curve 4 in Fig. 4). This implies the important role of solar wind heating of the subsolar ionosphere^{15,20,21} and the significant influence of transport processes within the ionosphere at large SZA¹².

The model of the subionopause pressure distribution with height and with the solar zenith angle seems to represent reasonably the observed behaviour of ionopause crossings at different SZA and allows one to obtain an 'instant' profile of the ionopause for given solar wind pressure.

Hydromagnetic dynamo in the cores of Uranus and Neptune

M. Torbett & R. Smoluchowski

Department of Astronomy, University of Texas, Austin, Texas 78712

The explanation of the origin of a magnetic field of Uranus is difficult because the structure of the planet's interior is not well known and the strong thermal flux, which is associated with the operation of hydromagnetic dynamos in Jupiter and Saturn, seems to be absent or very low. We show here that the composition, physical state and electrical conductivity of the planet's core permits the generation of a magnetic field within the very low observational limits of its heat emission. We also suggest that the higher density and higher pressures in the core of Neptune could explain the suspected absence of a measurable field on that planet even though it is a relatively strong source of heat.

While there are no rigorous theories which would permit us to make quantitative predictions of the magnetic fields of various planets it is usually possible to explain the presence or absence of these fields and sometimes even give a semiempirical estimate of their strengths¹⁻³. Observations⁴ of 0.5-MHz pulses analogous to jovian emission suggested that Uranus has a magnetic field but the planet had resisted efforts to explain its field's origin in a satisfactory manner. It now seems that a detailed consideration of convection and electrical conductivity in the planet's core indicates the presence of a thermally driven hydromagnetic dynamo.

The generation of magnetic fields in planets, whether by a thermally or precessionally driven dynamo, is very sensitive to details of the models of their interiors. The development of a satisfactory model of Uranus has been hampered by the availability of only approximate values of some of the important parameters, by the lack of good equations of state and by the difficulty in fitting, within reasonable limits, what is known about the chemical composition and evolution of the outer planets. In particular there is still an uncertainty of about a factor of two in the rotational velocity of the planet^{5,6}. This velocity strongly affects the structure of the outer parts of the planet and, to a much smaller degree, the size of the core. We have shown previously⁷ that a two-layer model of Uranus could account for the presence of a magnetic field only if the planet had a layer of metallic hydrogen or of a metallicity conducting molecular hydrogen and even then probably only if the dynamo were precessionally driven by Miranda. This conclusion was forced by the fact that in this model the core turned out to be solid while the layer containing liquid H_2O , NH_3 and so on had much too low an ionic electrical conductivity even though, as shown by experiment (W. J. Nellis, A. C. Mitchell, R. S. Hawke and R. K. Keeler, personal communication), the water would be pressure dissociated but not yet metallic. The above result was independent of the rate of rotation of the planet within the limits mentioned above.

The new three-layer model of Uranus of Hubbard and MacFarlane⁸ led us to review the situation as the model uses new equations of state and, within a wide range of rotational velocities, it seems to fit better the observational parameters as well as the chemical constraints. First, the model excludes the presence of a metallic or of a suitably conducting molecular hydrogen layer. Second, just as in the two-layer model, the liquid 'ice' layer has too low an ionic conductivity to permit the operation of a magnetic dynamo although a thin layer of 'metallic' H_2O could appear right at the core boundary. It remains then to analyse the planet's core which has a radius of 7,700 km

Received 10 March; accepted 24 April 1980.

- Smith, E. J., Davis, L. Jr, Coleman, P. J. Jr & Sonett, C. P. *J. geophys. Res.* **70**, 1571-1586 (1965).
- Dolginov, Sh. Sh., Eroshenko, E. G. & Zhuzgov, L. N. *Kosm. Issled.* **6**, 562-567 (1968).
- Dolginov, Sh. Sh., Eroshenko, E. G., Zhuzgov, L. N., Buzin, V. B. & Sharova, V. A. *Pisma Astr.* **2**, 88-90 (1976).
- Gringauz, K. I. *et al. in Physics of Solar Planetary Environments* (ed. Williams, D. J.) 918-932 (American Geophysical Union, Washington DC, 1976).
- Vaisberg, O. L. *et al. in Physics of Solar Planetary Environments* (ed. Williams, D. J.) 904-917 (American Geophysical Union, Washington DC, 1976).
- Science* **203**, 743-808 (1979).
- Science* **205**, 41-121 (1979).
- Bauer, S. J., Donahue, T. M., Hartle, R. E. & Taylor, H. A. *Jr Science* **205**, 109-111 (1979).
- Kliore, A. J., Woo, R., Armstrong, J. W., Patel, I. R. & Croft, T. A. *Science* **203**, 765-768 (1979).
- Knudsen, W. C. *et al. Science* **203**, 757-759 (1979).
- Taylor, H. A. *Jr et al. Science* **203**, 752-754 (1979).
- Taylor, H. A. *Jr et al. Science* **203**, 755-757 (1979).
- Russell, C. T. *J. geophys. Res.* **82**, 625-631 (1977).
- Russell, C. T., Elphic, R. C. & Slavin, J. A. *Science* **203**, 745-748 (1979).
- Brace, L. H. *et al. Science* **203**, 763-765 (1979).
- Elphic, R. C., Russell, C. T., Slavin, J. A., Brace, L. H. & Nagy, A. F. *Geophys. Res. Lett.* (submitted).
- Spreiter, J. R., Alksne, A. Y. & Summers, A. L. in *Physics of the Magnetosphere* (eds. Carovillano, R. L., McClay, J. F. & Radoski, M. R.) 304-378 (Reidel, Dordrecht, 1968).
- Spreiter, J. R., Summers, A. L. & Rizzi, A. W. *Planet. Space Sci.* **18**, 1281-1290 (1970).
- Russell, C. T., Elphic, R. C. & Slavin, J. A. *Proc. Magnetospheric Boundary Layer conf.* 231-239 (ESA SP-148, 1979).
- Scarf, F. L., Taylor, W. W. L. & Green, J. M. *Science* **203**, 748-750 (1979).
- Knudsen, W. C. *et al. Science* **205**, 105-107 (1979).
- Ivanov-Kholodny, G. S. *et al. Soviet Phys. Usp.* **20**, 1025-1027 (1977).
- Ivanov-Kholodny, G. S. *et al. 21st COSPAR Meet.*, Innsbruck, (1978).

($r_c/R_u \sim 0.3$), and contains 38, 25, 25 and 12% of SiO_2 , MgO , FeS and FeO respectively. The melting temperatures of these compounds as a function of radius in the core were calculated using the same method as in our previous paper (assuming the Grüneisen parameter to be the same for FeO and FeS) and the densities at which these compounds should become metallic were estimated using Herzfeld's molar refractivity criterion⁹. The results are shown in Figs 1 and 2. It seems that in an isothermal core FeS and FeO are always liquid, MgO is always solid while SiO_2 is solid in the inner part of the core. FeO and FeS are metallic throughout the core, the sulphide being known to change from a low gap semiconductor to a metal at pressures of the order of kilobars¹⁰. MgO and SiO_2 are metallic up to $\sim 85\%$ of the radius of the core.

It can be shown that initial gravitational condensation and radioactive heating led to a temperature of the interior of Uranus which was probably in excess of that required to keep the core metallic and entirely liquid. The outward flow of this heat produced convection which kept the core well mixed during this early high temperature phase. As the core cooled, however, large differences in the density of its constituents produced gravitational, diffusion controlled, separation between the heavy iron compounds and other lighter constituents which led to a significant evolution of additional heat. Further cooling of the core resulted in the formation of solid MgO which floated towards the surface of the core thereby releasing energy which in turn slowed down the cooling and the segregation. This self-regulating aspect of the process, reminiscent of that proposed¹¹ for the H-He system in the giant planets and necessary to explain the present magnetism and excess luminosity of Saturn¹², may be still active because the theory is certainly not rigorous enough to ensure that the melting temperature of MgO near the surface of the core is indeed 1,000 K higher than the calculated temperature at this radius. It seems reasonable, therefore, to conclude that even now the core of Uranus is not isothermal but adiabatic. Unfortunately, lack of knowledge of the phase diagram of the multicomponent alloy and especially of mutual solubilities of the components in the early stages of the evolution of the planet makes a quantitative prediction of the rate of heat generation in the core almost impossible. The total energy available from gravitational interchange in the core can be estimated, however, to be $\sim 10^{39}$ erg which distributed over a time scale of 10^{10} yr yields an average flux of the order of $40 \text{ erg cm}^{-2} \text{ s}^{-1}$ at the surface of the planet, a value compatible

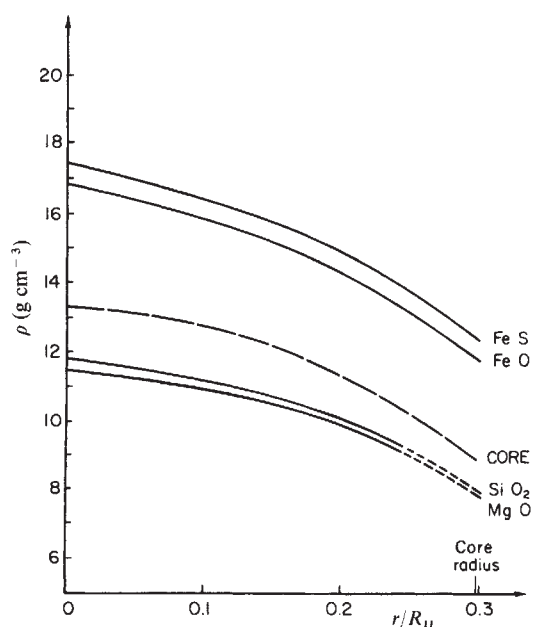


Fig. 1 Density profiles of the core and of its constituents: solid line, metallic; dashed line, non-metallic. R_U is the radius of the planet.

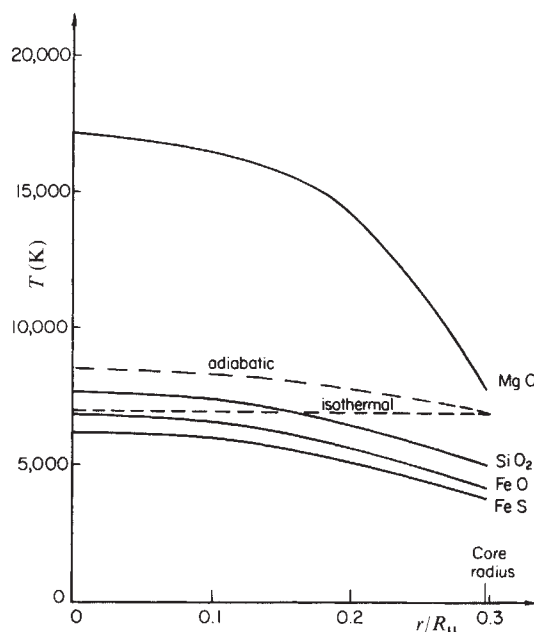


Fig. 2 Melting temperature profiles of the constituents of the core (solid line) and the temperature gradients in the core (dashed lines). R_U is the radius of the planet.

with the observational¹³ upper limit for Uranus of about $10^2 \text{ erg cm}^{-2} \text{ s}^{-1}$. The total original primordial energy of the planet was probably not less than 10^{40} erg. The range of possible compositions of cores of the giant planets introduces an uncertainty of about a factor of two into the above estimates.

Assuming a superadiabaticity of $\nabla - \nabla_s = 10^{-6}$, a thermal expansion coefficient of the order of 10^{-6} and a mixing length $l \sim 10^{-3} r_c$ one obtains for the convective velocity in the core $v \sim 10^{-1} \text{ cm s}^{-1}$. In view of the highly heterogeneous chemistry of the core it seems reasonable to put electrical conductivity $\sigma = 10^{-5} \text{ e.m.u.}$ which is typical of low conductivity metals. With these values for v and σ , the necessary condition for the operation of a thermally driven hydromagnetic dynamo—the magnetic Reynolds number, $R = \sigma \mu L v$ where μ is the permeability and L is the size of the core, has to be greater than 10—is amply satisfied. The heat flux carried by this convection, as estimated using the expression $\Phi = \rho C_p v l T (\nabla - \nabla_s) (2H_p)^{-1}$ (Scalo, personal communication) is about $10 \text{ erg cm}^{-2} \text{ s}^{-1}$ at the surface of the planet. Here H_p is the pressure scale height and C_p is the specific heat, about $5 \times 10^6 \text{ erg g}^{-1} \text{ deg}^{-1}$.

The presence of an internal heat source in the core implies, of course, that the central temperature of the planet is somewhat higher than in the original model⁹, but this does not significantly affect the model's other features because the presence of a comparable flux is already taken into account and the thermal pressure is negligible. The approximate adiabatic gradient is also indicated in Fig. 2 showing that, except for MgO , all constituents of the core are liquid. One may add that Busse's semi-empirical rule² applied to Uranus' core leads to a surface field of the order of 10^{-1} G .

Applying the same kind of reasoning to the three-layer model of Neptune⁸ one concludes that because of higher densities and pressure nearly half of the volume of its core is solid as compared to about one-third on Uranus. Thus one may expect the differentiation, the heat evolution, the rate of convection and the magnetic field of the core to be much lower than on Uranus. On Neptune, just as on Uranus, the ionic conductivity of the liquid 'ice' layer is too low and the probable layer of 'metallic' H_2O at the core boundary too thin to permit the operation of a hydromagnetic dynamo even in the presence¹⁴ of the high heat flux.

It seems, therefore, that a slightly modified three-layer model of Uranus explains in a natural way the presence of a magnetic

field on this planet and does not require making rather problematic assumptions which were necessary for the two-layer model. Application of a similar analysis to the three-layer model of Neptune explains its very low, if any, magnetic field in spite of the high thermal flux.

This work was supported by NASA grant NSG 7505 Sup. 1.

Received 11 February; accepted 23 May 1980.

1. Stevenson, D. J. *Icarus* **22**, 403 (1974).
2. Busse, F. H. *Phys. Earth planet. Inter.* **12**, 350 (1976).
3. Smoluchowski, R. *Phys. Earth planet. Inter.* **20**, 247 (1979).
4. Brown, W. L. *Astrophys. J.* **207**, L209 (1976).
5. Dunham, E. & Elliot, J. L. *Bull. Am. astr. Soc.* **11**, 568 (1979).
6. Smith, H. J. & Slavsky, D. B. *Bull. Am. astr. Soc.* **11**, 568 (1979).
7. Torbett, M. & Smoluchowski, R. *Geophys. Res. Lett.* **6**, 675 (1979); *Lunar planet. Sci.* (in the press).
8. Hubbard, W. B. & MacFarlane, J. J. *J. geophys. Res.* **85**, 225 (1980).
9. Ross, M. J. *chem. Phys.* **56**, 4651 (1972).
10. Stiller, H., Siebold, U. & Vollstaedt, H. *Fiz. Svoistva Gorn. Porod. Miner. Vys. Temp. Mater. Vses. Soveshch.* **4**, 198 (1974).
11. Salpeter, E. E. *Astrophys. J.* **181**, L183 (1973).
12. Stevenson, D. J. *Science* **208**, 746 (1980).
13. Hubbard, W. B. *Icarus* **35**, 177 (1978).

Solar cycle changes in the polar solar wind

W. A. Coles*, B. J. Rickett*, V. H. Rumsey*,
J. J. Kaufman*, D. G. Turley*, S. Ananthakrishnan†,
J. W. Armstrong‡, J. K. Harmons§, S. L. Scott||
& D. G. Sime¶

*Department of Electrical Engineering and Computer Sciences, University of California, San Diego, La Jolla, California 92093

† Radio Astronomy Centre, Tata Institute for Fundamental Research, Ootacamund 643001, India

‡ Jet Propulsion Laboratory, 4800 Oak Grove Drive, Pasadena, California 91103

§ Arecibo Observatory, Arecibo, Puerto Rico 00612

|| Owen's Valley Radio Observatory, Box 387, Big Pine, California 93513

¶ High Altitude Observatory, National Center for Atmospheric Research, PO Box 3000, Boulder, Colorado 80307

Although the 11-yr cycle of solar activity was first recognized in 1843 (ref. 1) and was subsequently traced back to the seventeenth century²⁻⁴, it is still only poorly understood. The most obvious indicators of the activity cycle are sunspots, flares, plagues, and so on. The corona as seen during an eclipse also reflects the cycle, appearing flattened at the poles near solar minimum and almost spherical near the solar maximum. All such phenomena are intimately linked to the solar magnetic fields, which exhibit complex but systematic variations. Whereas there are satisfactory qualitative models (see refs 5, 6) for the magnetic variations, the underlying physics is still obscure. The solar cycle also influences the Earth causing, for example, changes in geomagnetic activity, the magnetosphere and aurorae, the ionosphere, the flux of galactic cosmic rays and possibly the weather. These modulations are not well understood but must be transmitted by one or more of the following: electromagnetic radiation; solar wind thermal plasma and its magnetic field; energetic particles. We here present new observations on the changing three-dimensional form of the solar wind, which helps to relate some of the modulations.

The solar wind also shows the effects of the solar cycle, but changes observed by ecliptic spacecraft are minor compared with the dramatic changes observed in the corona for example. Two processes are apparent in the observations of 1962–74 (ref. 7). First, at sunspot maximum, there is an increase in flare-associated transient disturbances in the solar wind. Second the quasi-static structures of the solar wind such as magnetic sectors and high speed streams are much more evident and stable during the declining and minimum years of activity. In fact the

dominance of the high speed streams during the declining phase of the cycle is the most obvious change in the ecliptic solar wind. We show that an understanding of the three-dimensional wind structure explains the occurrence of such streams.

Direct spacecraft observations have reached $\pm 16^\circ$ in latitude although most are confined to $\pm 10^\circ$. These show very high latitude gradients of velocity during streams^{8,9}, and they have helped to clarify the magnetic sector structure^{3,4} but they are obviously limited in defining the overall latitude structure. However, the indirect techniques of interplanetary scintillation (IPS) (refs 10–13) and comet-tail observations¹⁴ can cover essentially all latitudes albeit with reduced precision. IPS observations from 1971 to 1979 show that the solar wind does respond dramatically to the solar activity cycle but the primary effects are in the polar regions.

The IPS method, pioneered by Dennison and Hewish¹⁵ relies on three spaced antennas to record signals from a small diameter radio source. The scintillations in the strength of the signals at each antenna are caused by inhomogeneities in the solar wind; the signals are cross-correlated to yield time offsets, from which the speed of the scintillation pattern can be deduced.

The UCSD observatory, which operates at 74 MHz, and the methods of data analysis have been described elsewhere^{16,17}. A careful comparison of ecliptic IPS observations with spacecraft observations¹⁸ has 'calibrated' the IPS method. From this work we conclude that the IPS-observed speed is within $\pm 50 \text{ km s}^{-1}$ of the solar wind speed at the point where the radio-wave scattering is strongest. We thus adopt the simplified interpretation that the IPS speed gives an estimate of the solar wind speed at that single point in space. We make IPS observations of eight radio sources daily, from which we typically obtain useful solar wind speed estimates at 3–5 such effective locations. The heliographic latitude of these locations spans 60°S to 70°N . However, the coverage is not continuous; northern latitudes ($> 20^\circ$) are observed from March–July and October–November, while southern latitudes ($> 20^\circ$) are observed June–July, each year (see Fig. 1 of ref. 19).

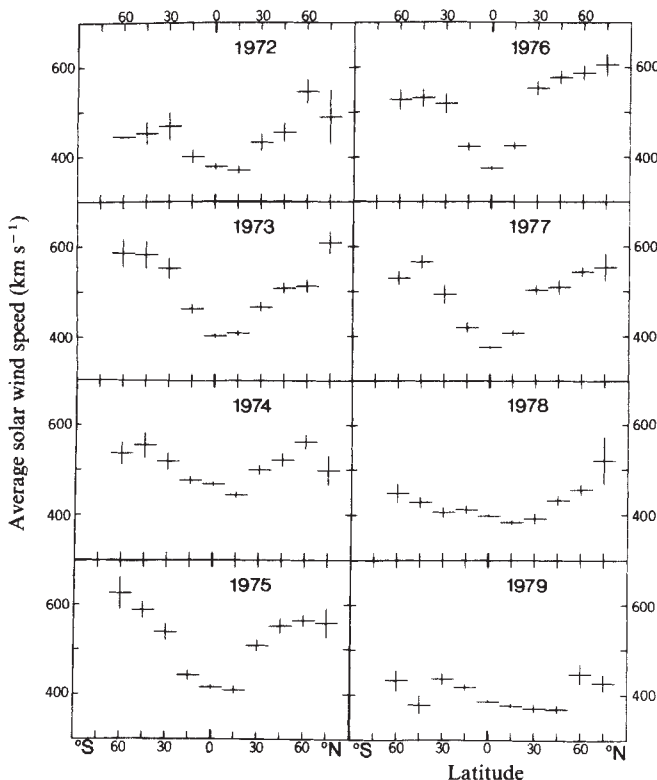


Fig. 1 Solar wind speed measured by IPS averaged into 15° intervals in heliographic latitude for the years 1972–79. The error bars are $\pm$ twice the standard deviation in the average.

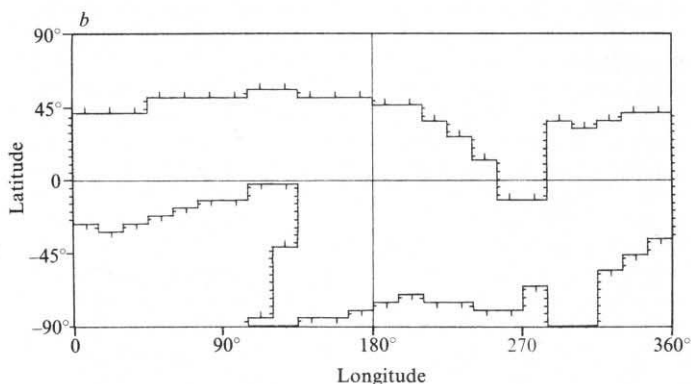
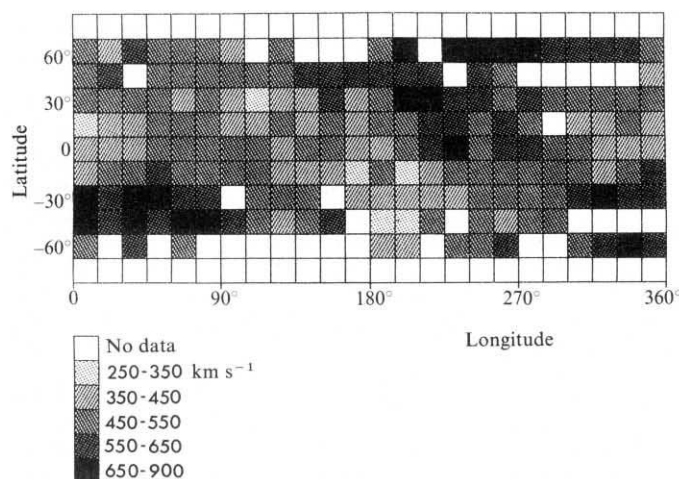


Fig. 2 *a*, The average solar wind speed in $15^\circ \times 15^\circ$ bins of heliographic latitude and longitude coded by the grey scale as indicated. The IPS data were averaged for Carrington rotations 1612–1617 (March–July 1974). *b*, Coronal holes indicated by a contour of low K -coronameter polarization brightness (1.5×10^{-8} pB); the tick marks point towards low brightness, that is towards the holes. Data, supplied by Drs R. and S. Hansen, are averaged for the same period as *a*.

The solar wind speed can be averaged over longitude to provide the speed versus latitude plots of Fig. 1. Their characteristic U-shape shows that the wind speed is on average considerably faster at high latitudes than at the solar equator²⁰. This phenomenon is best described as evidence for persistent fast polar streams from the north and south poles. In studying the long-term changes in Fig. 1, we must be aware of the reduced number of observations at high latitudes, with a corresponding increase in the error bars with latitude. Nevertheless, we can identify three phases in the evolution of the polar streams.

First, during the years of declining activity 1973–75, equatorial speeds are somewhat elevated and there is a gradual increase of speed between 0° and $\pm 60^\circ$. The distribution of mean wind speed with latitude and longitude averaged over six rotations is shown in Fig. 2*a*. Here it is apparent that the polar streams are tilted from the axis, remaining opposite each other but centred near latitudes of $\pm 50^\circ$. These streams are wide enough to reach the ecliptic, where they are seen as two fast streams per rotation and also cause an increase in the mean speed⁷. Figure 2*b* shows that these fast streams come from coronal holes, where the polar holes are shown to be tilted in much the same way.

The second phase, 1976–77 (Fig. 1), shows slower equatorial speeds and a sharp transition from slow to fast between 15° and 30° in both the north and south. This corresponds to a better alignment of the polar streams with the rotation axis, as revealed in latitude–longitude plots²¹. Note that the gradient of the mean speed is greater than 10 km s^{-1} per degree between 15° and 30° . This is comparable with instantaneous measurements of the latitude gradient made with two spacecraft (see refs 8, 9). The polar holes were also wide and axially aligned during these years of solar minimum as shown by K -coronameter data²² and by eclipse photographs which are elongated along the equator due to low densities over the poles²³.

The third phase, 1978–79, shows a flattening of the U-shape curve, that is a dramatic reduction in the wind speed at 30 – 60° N and S, implying that the polar streams are much narrower. Note that this change probably occurred at the same phase of the previous cycle. Hewish and Symonds¹⁰ observed higher speeds at north latitudes than at the equator in 1966, but not in 1967. Their restricted data coverage meant they could not draw any strong conclusions, but it is now clear that this is the same change which we see 11 yr later.

The polar holes also contracted during 1977, as the new sunspots emerged at mid-latitudes. This implies that bipolar magnetic regions are dominating the mid-latitudes restricting the extent of the open field regions (polar holes). Figure 3 compares average wind speed at 0 , 30° and 60° with the area of the polar holes from 1971 to 1979. Hole area estimates from

observations of He $10,830 \text{ \AA}$ made at Kitt Peak are plotted for 1974–78, together with earlier estimates by various techniques²⁴. The areas are given as percentages of $4\pi \text{ sr}$ at the photosphere. The slowing of the wind at 30° and 60° north and south in late 1977 to the present (a narrowing of the polar streams), is closely matched by the shrinking of the polar holes.

The latitude boundary of the polar holes and polar streams are compared in Fig. 4 (assuming axial symmetry). The stream boundary is chosen to be the latitude at which the velocity is 500 km s^{-1} . There are clearly large changes in the width of the polar streams. Between 1974 and early 1977, the polar stream boundaries were near $\pm 30^\circ$ showing that 50% of $4\pi \text{ sr}$ were occupied by solar wind with an average wind speed $> 500 \text{ km s}^{-1}$. By contrast, from late 1977 to the end of 1979, the polar stream boundaries moved up to $\pm 65^\circ$, showing that the fast polar wind occupied only 9% of $4\pi \text{ sr}$.

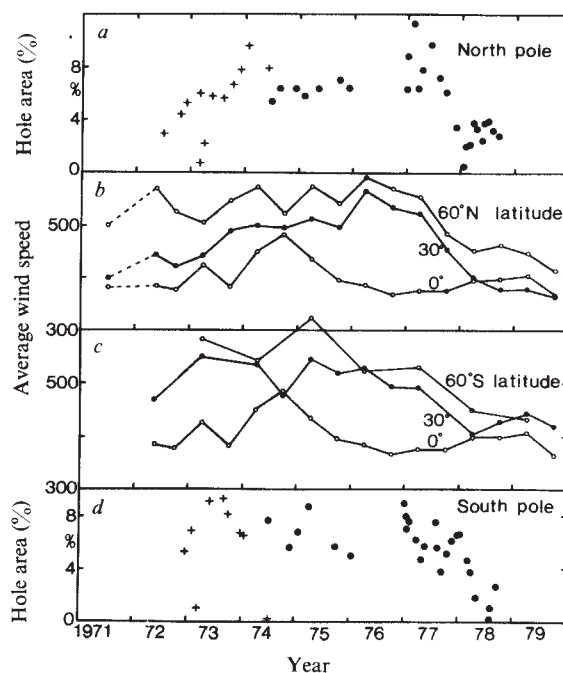


Fig. 3 The solar wind speeds averaged into 15° latitude intervals for six month periods are plotted against time in *b* and *c*. Single rotation estimates of polar coronal hole areas derived from photospheric observations are plotted for the north polar hole in *a* and for the south in *d*. Symbols indicate techniques from ref. 24. The slowing of the wind above $\pm 30^\circ$ latitude in 1978 is matched by a narrowing of the polar holes.

When these changes in the fast polar streams are compared with changes in the polar coronal holes, it is apparent that the polar hole boundaries do not change as much as the polar stream boundaries. Although there are real variations between rotations, the mean hole boundaries remain at $\pm 60^\circ$ (13% solid angle) through early 1977, shrinking to $\pm 70^\circ$ (6% solid angle) in 1978. Data are not yet available for 1979. The ratio of the solid angle for fast polar streams to that for coronal holes defines an expansion factor which characterizes the degree to which the coronal holes open out faster than radial as they become fast streams. This ratio decreased from 3.8 for 1974–77 to 1.5 for 1978. Whereas the numerical value of the ratio cannot be easily related to the areal expansion factor as defined and measured for a northern polar hole²⁵, the change in the ratio indicates that the areal expansion factor of the polar holes decreased substantially as the holes narrowed. A large expansion factor implies that the solar wind is accelerated to supersonic speeds closer to the base of the corona than for a smaller areal expansion²⁶.

The agreement with IPS observations in 1967 and 1968, and the agreement with the polar hole variations shows that the changes in the fast polar stream are part of the solar cycle. Studies of polar faculae over many solar cycles²⁷ suggest that the polar holes narrow for several years at the peak of activity. The polar magnetic field polarity also reverses near solar maximum. Clearly the polar holes; defined as unipolar open field regions, must undergo a major change and disappear altogether when the fields reverse. Eclipse photographs tell a similar story. The flattened images at solar minimum indicate the large low density holes over the polar regions; the circular images at solar maximum indicate no such polar holes. The changing three-dimensional solar wind structure reported here helps explain the solar cycle modulations of several phenomena.

The average level of geomagnetic activity varies with solar cycle but the pattern shows differences from one cycle to the next. Two mechanisms are thought to be responsible²⁸: the increase in solar wind transients near the maximum phase of the cycle, and the increase of ecliptic fast streams in the declining phase. Both processes cause enhanced geomagnetic activity index. However, their relative importance change from cycle to cycle, so that the peak index sometimes occurs near the maximum phase, sometimes in the declining phase, or sometimes extends between the two. In particular, it has been suggested²⁹ that the previous solar cycle (number 20) was unusual in the dominance of ecliptic streams and associated geomagnetic activity during the declining phase. Such cycle-to-cycle variability is easier to understand in the context of the three-dimensional stream structure. The recurrent ecliptic streams are seen when the long-lived polar streams are tilted far enough from the axis to intersect the ecliptic. For obscure reasons, such tilting is greatest in declining solar activity. However, we suggest that the degree of tilt varies from one cycle to the next causing recurrent ecliptic streams and accompanying geomagnetic activity to be more pronounced in some 11-yr cycles than in others.

The average flux of galactic cosmic rays detected at the Earth is also modulated by the sunspot cycle, such that minimum cosmic ray flux occurs at sunspot maximum and vice-versa³⁰. The spherically symmetrical diffusion models for this modulation predict an 11-yr variability in the strength of inhomogeneities in the magnetic field and also a radial gradient in the cosmic ray flux, both of which are now contradicted by observations³¹. The present results indicate a need for models where the magnetic geometry in three-dimensions is important (such as those of Jokipii *et al.*,³²), especially when combined with the recent correlation of polar hole area and cosmic ray flux³³. We suggest that at solar minimum wide polar holes connect to wide fast polar streams, which are presumably unipolar and may in turn be connected to the interstellar magnetic field, allowing easy access of cosmic rays. At solar maximum the polar holes are constricted by high latitude active regions, causing a narrowing of the polar streams, that is a smaller area of easy access which reduces the cosmic ray flux.

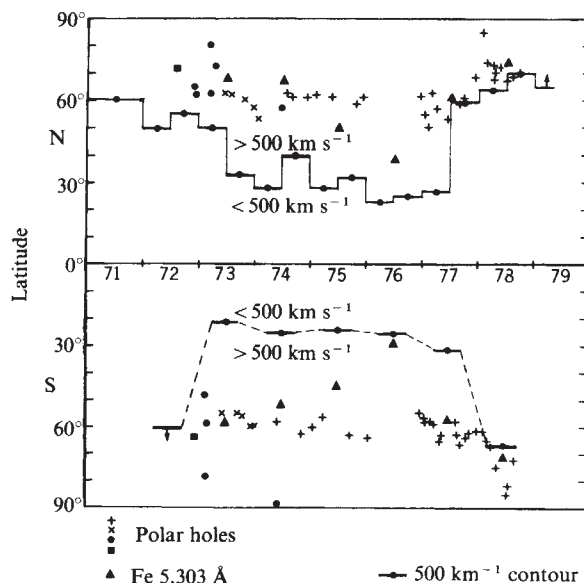


Fig. 4 Solid curve (in the north) and horizontal bars (in the south) indicate the contour of 500 km s^{-1} average solar wind speed. The latitude boundaries of the polar holes are indicated by various symbols: +, He 10,830 Å; x, different techniques (see ref. 24); ▲, Fe 5,303 Å (threshold at 1.5×10^{-6} of disk centre brightness, data from Sacramento Peak Observatory).

Fast solar wind streams from the polar coronal holes persist for at least 6 yr but contract as the solar activity cycle approaches maximum. The polar coronal holes are observed to narrow at the same time, presumably in response to the changing solar magnetic field. The link between coronal holes and fast wind streams is thus extended to polar latitudes and to a major fraction of the 11-yr cycle. This picture throws considerable light on the problem of solar cycle modulation of galactic cosmic rays, geomagnetic activity and the presence of fast ecliptic wind streams.

This research has been supported from the start by the Atmospheric Sciences Division of the NSF (ATM 78-6770), and since 1977 by the A. F. Geophysical Laboratories (F19628-77-C-0161).

Received 14 March; accepted 8 May 1980.

- Schwabe, H. *Astr. Nachr.* **20**, (1843).
- Wolf, R. *Astr. Mitt. Zürich* **1**, 8 (1856).
- Maunder, E. W. *Mon. Not. R. astr. Soc.* **50**, 251–260 (1890).
- Eddy, J. A. *Science* **192**, 1189–1192 (1976).
- Babcock, H. W. *Astrophys. J.* **133**, 572–587 (1961).
- Leighton, R. B. *Astrophys. J.* **156**, 1–41 (1969).
- Gosling, J. T., Asbridge, J. R., Bame, S. J. & Feldman, W. C. *J. geophys. Res.* **81**, 5061 (1976).
- Rhodes, E. J. & Smith, E. J. *J. geophys. Res.* **80**, 917–928 (1975).
- Schwenn, R. J. *J. geophys. Res.* **83**, 1011 (1978).
- Hewish, A. & Symonds, M. D. *Planet. Space Sci.* **17**, 313–320 (1969).
- Vitkevich, V. V. & Vlasov, V. I. *Soviet Astr.* **13**, 669–676 (1970).
- Coles, W. A. & Maagoe, S. J. *J. geophys. Res.* **77**, 5622–5624 (1972).
- Watanabe, T., Shibasaki, K. & Kakinuma, T. *J. geophys. Res.* **79**, 3841–3843 (1974).
- Brandt, J. C., Harrington, R. S. & Rosen, R. G. *Astrophys. J.* **196**, 877–878 (1975).
- Dennison, P. A. & Hewish, A. *Nature* **213**, 343–346 (1967).
- Armstrong, J. W. & Coles, W. A. *J. geophys. Res.* **77**, 4602–4609 (1972).
- Coles, W. A. & Kaufman, J. J. *Radio Sci.* **13**, 591–597 (1978).
- Coles, W. A., Harmon, J. K., Lazarus, A. J. & Sullivan, J. D. *J. geophys. Res.* **83**, 3337–3341 (1978).
- Coles, W. A. & Rickett, B. J. *Solar Geophysical Data, Explanation of Data Reports*, No. 390, 13–15 (1977).
- Coles, W. A. & Rickett, B. J. *J. geophys. Res.* **81**, 4797–4799 (1976).
- Sime, D. G. & Rickett, B. J. *J. geophys. Res.* **83**, 5757–5762 (1978).
- Hundhausen, A. J. in *Proc. Symp. on Solar Cycle from Space*, Wellesley (NASA, 1979).
- Allen, C. W. *IAU Symp. on Solar Corona*, 241 (1961).
- Sheeley, N. R. Jr in *Proc. Symp. on Solar Cycle from Space*, Wellesley (NASA, 1979).
- Munro, R. H. & Jackson, B. V. *Astrophys. J.* **213**, 874–876 (1977).
- Kopp, R. A. & Holzer, T. E. *Solar Phys.* **49**, 43–56 (1976).
- Sheeley, N. R. Jr *J. geophys. Res.* **81**, 3462–3464 (1976).
- Crooker, N. U., Feynman, J. & Gosling, J. T. *J. geophys. Res.* **82**, 1933–1937 (1977).
- Gosling, J. T., Asbridge, J. R. & Bame, S. J. *J. geophys. Res.* **82**, 3311–3314 (1977).
- Pomerantz, M. A. & Duggal, S. P. *Rev. Geophys. Space Sci.* **12**, 343–357 (1974).
- Fisk, L. A. *Solar System Plasma Physics* (Reidel, Dordrecht 1979).
- Jokipii, J. R. & Kopriva, D. A. *Astrophys. J.* **234**, 384–392 (1979).
- Hundhausen, A. J., Sime, D. G., Hansen, R. T. & Hansen, S. F. *Science* **207**, 761–762 (1980).
- Smith, E. J., Tsurutani, B. T. & Rosenberg, R. L. *J. geophys. Res.* **83**, 717 (1978).

Peninsula method of ULF signal generation

A. C. Fraser-Smith & O. G. Villard Jr

Radioscience Laboratory, Stanford Electronics Laboratories,
Stanford University, Stanford, California 94305

Natural pulsations of the geomagnetic field in the ultra-low-frequency range (ULF; <5 Hz) have been studied for many years, but progress has been limited by the lack of controlled experiments on their generation and propagation. Clearly, a method for producing the pulsations artificially on a controlled basis would contribute greatly to our understanding of their properties. In 1975 we participated in a theoretical study¹ that described a method for generating artificial Pc 1 geomagnetic pulsations (0.2–5 Hz) by driving a large alternating electric current with frequency in the Pc 1 range around a relatively nonconducting peninsula in an enclosed sea or large saline lake. The study predicted that the alternating magnetic field of the current loop in the sea could disturb the lower ionosphere above the peninsula and that hydromagnetic (HM) waves at the Pc 1 frequency would propagate away from the disturbed region to large distances in the ionosphere and magnetosphere. Two important conditions for the generation to take place were that the amplitude of the ULF magnetic field fluctuations should be greater than ~ 1 nT (refs 2, 3) and that suitable propagation conditions should exist for the HM waves. We report here the results of two experiments on the 'peninsula method' of Pc 1 pulsation generation conducted during 1975–76. Our results, when combined with those from a recent peninsula experiment by Soviet scientists^{4,5}, strongly support the proposed method of Pc 1 pulsation generation.

Our peninsula experiments used the North Neck Peninsula on Chappaquiddick Island, Massachusetts (Fig. 1), which is located in an enclosed body of seawater (enclosure of the peninsula provides well defined boundary conditions and protection from wave action; it is not a necessary condition for ULF signal generation). During the summer of 1975 we carried out a passive experiment using the seawater around the peninsula as a receiving loop. Two electrodes constructed of thin copper sheet ~ 0.45 m square were inserted in the water on either side of the peninsula and connected by shielded cables (total length, 300 m; total resistance, $\sim 10 \Omega$) to an electronics package consisting of a ULF differential amplifier, a timer, a calibrator and a low pass filter with an overall voltage gain of 200 at a frequency of 1 Hz. The resistance of the cable-seawater-cable circuit as seen by the electronics package was $\sim 12 \Omega$, implying that the resistance between the two electrodes was roughly 2Ω . The output of this system, which had a flat frequency response over the Pc 1 frequency range, was recorded on a slow-speed analogue magnetic tape recorder.

Measurements of ULF geomagnetic pulsation activity were made each night between 26 August and 5 September, and several well defined Pc 1 events were recorded. A spectrogram of one particularly long-lasting and large-amplitude event is shown in Fig. 2, together with spectrograms of the same event as seen at Roberval, Quebec, and at Stanford, California (see Fig. 3 for locations of the stations). Although this was a preliminary experiment, and some desirable controls were necessarily lacking, it demonstrated that Pc 1 pulsations could be measured across the neck of a peninsula. Further, the high signal-to-noise ratio of the measurements, as shown by the data in Fig. 2,

supported the peninsula current loop concept, as a large receiving area would be required to provide the high ratio.

An exploratory small-scale active experiment was conducted during the summer of 1976. Electrodes were installed at roughly the same locations on either side of North Neck Peninsula as during 1975, but this time they were larger and were constructed of galvanized iron pipe. They were spaced ~ 180 m apart and connected to a ULF current source by heavy-gauge insulated aluminium wire (wire diameter 0.52 cm), and the resistance of the current path was 0.4Ω , as compared with 12Ω the previous year. The ULF current source used four automobile starter relays in a bridge circuit to produce a pseudo sine wave alternating current; it could deliver a maximum current of 60 A into the 0.4Ω load, and its frequency was adjustable in the range 0.5–12 Hz. Power was provided by two 12-V automobile batteries connected in series.

There were three parts to this active experiment: (1) a fluxgate magnetometer was used to measure the magnetic field produced on the peninsula and its vicinity; it had a flat frequency response from d.c. to 500 Hz, and its noise level was ~ 1 –2 nT in the 0.2–5 Hz band; (2) a three-axis electric field (or current) probe was constructed and used to measure the ULF currents flowing in the seawater around the peninsula. It had three orthogonal pairs of contacts spaced 0.5 m apart, and each pair fed a preamplifier with a voltage gain of 100 and a bandpass of 0.5–5 Hz.

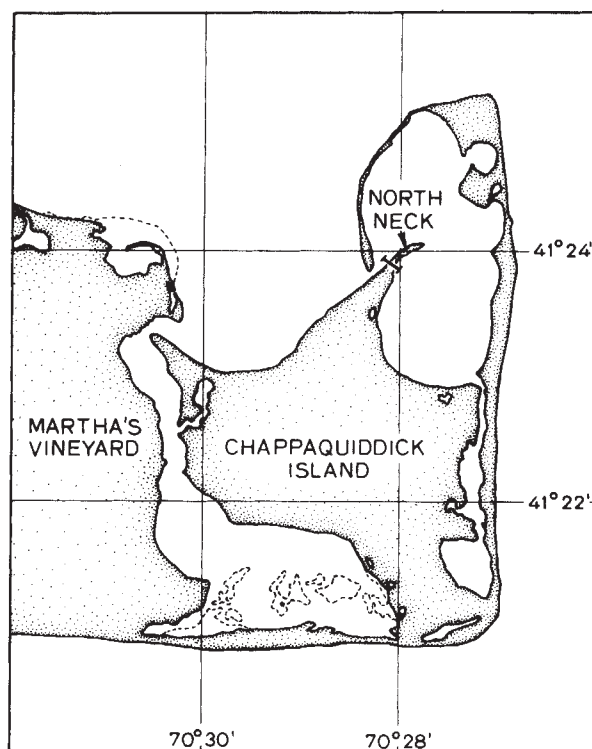


Fig. 1 Map of Chappaquiddick Island, Massachusetts. The experiment was conducted on North Neck, which projects into Cape Poge Bay (upper right), and the location of the electrodes on either side of the Neck is superimposed on the map. The longitude lines are 2.8 km apart.

The calibrated outputs from the preamplifiers were separately monitored on a portable chart recorder, and the minimum electric field amplitude that could be measured was about 1 mV m^{-1} . (3) Finally, measurements of the magnetic field produced above the peninsula were made with the total field magnetometer on a P-3C Orion aircraft.

The magnetic field measurements on the peninsula were largely dominated, as expected, by the field of the electrode feed wires. This field was calculated, and it was found that the calculated values were $\sim 20\%$ greater than the measured values. The difference can be attributed to current flow directly through and under the peninsula. Noise caused by boat motion limited the probe measurements to points within ~ 300 m of the electrodes, and the principal result of these measurements was finding that the current flow was largely radial at all distances where measurements were possible. This finding indicated that some of the current paths would enclose a large area: a necessary condition for a large magnetic moment for the 'peninsula antenna'. Further evidence for this latter condition was provided by the aircraft measurements, which were made during eight passes over the peninsula at altitudes in the range 160–320 m.

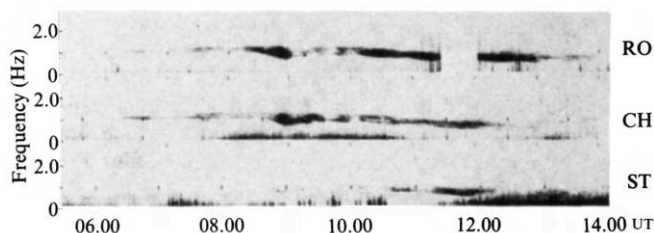


Fig. 2 Spectrograms of a long-lasting Pc 1 geomagnetic pulsation event, as recorded at Roberval, Quebec (RO), Chappaquiddick Island, Massachusetts (CH), and at Stanford, California (ST), on 2 September 1975. The recording at Chappaquiddick Island was made with a 'peninsula antenna'; the other recordings were made with conventional ULF solenoid antennas. A power failure caused the blank interval in the Roberval record.

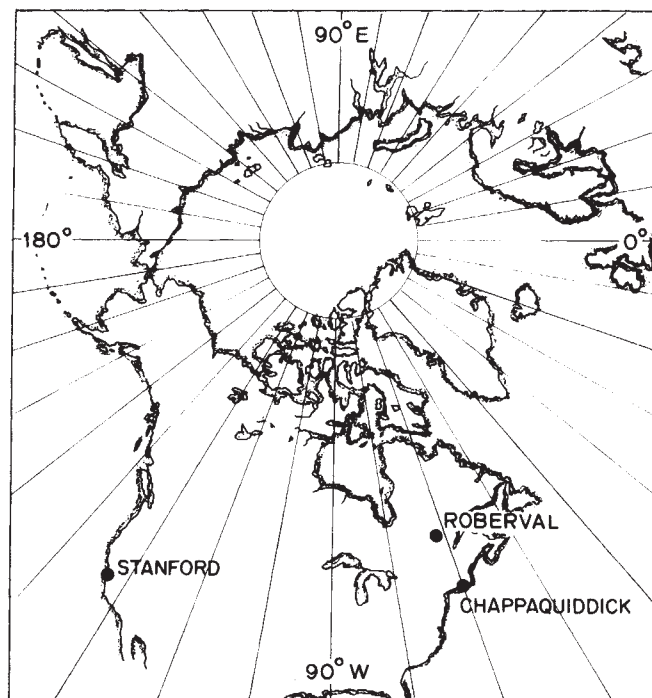


Fig. 3 Map of North America, showing the relative geographic locations of the ULF recording stations at Chappaquiddick, Massachusetts; Roberval, Quebec; and Stanford, California.

The sensitivity of the magnetometer was reduced after each pass, and even on the last pass, with minimum sensitivity (measurement range 0–40 nT), the peninsular current loop produced off-scale readings.

Although these experiments supported the feasibility of the peninsular current loop concept, they only gave indirect information about the magnetic fields produced in the ionosphere. We therefore computed the amplitude of the magnetic field produced at an altitude of 100 km above the peninsula during our active experiment, using a standard finite difference relaxation method to obtain the electric potentials and current distribution in the seawater and then the Biot-Savart law for the magnetic field. The amplitude obtained was 0.017 ± 0.008 pT. This is about 49 times larger than the field that would be produced by the same total current flowing through a wire laid along the shoreline of the peninsula from one electrode to the other. Assuming a 20% loss of current through the peninsula (as suggested by our magnetic field measurements) the field produced by the peninsula-seawater combination is still ~ 35 –40 times larger than the comparison value. Note that this comparison does not take into account the greater power that would be required to drive the current through the wire outlining the peninsula.

An experiment was recently reported by Soviet scientists in which a large current produced by a portable magnetohydrodynamic (MHD) pulse generator was driven through the sea surrounding the Rybachiy Peninsula (69°N , 32.5°E), and the resulting magnetic field was measured over the adjacent Kola Peninsula^{4,5}. The maximum duration of the current pulses was ~ 6 s, and measurements showed that the equivalent magnetic moment of the current loop around the peninsula was 10^{14} A m². This loop was reported to take an equivalent area of about 5,000 km², implying that the amplitude of the current was 20 kA. As the resistance of the loop was 90 m Ω (this includes the

resistance of the cables joining the MHD generator to the sea on either side of the peninsula; these cables weighed 160 tons), a power of about 36 MW was required. Measurements of the magnetic field were made at distances up to 750 km from the peninsula, and at 320 km the electric and magnetic fields had amplitudes of about 0.01 V m⁻¹ and 1 nT, respectively.

These results are in good agreement with the predictions of Lipa *et al.*¹. Note the very small resistance of the peninsula current loop and the resulting large current (and loop magnetic moment) for a moderate power. They imply that a substantial (≥ 2 nT) ULF magnetic field can be produced in the lower ionosphere above the peninsula: one of the primary requirements for the artificial generation of Pc 1 pulsations. Unfortunately, the Soviet experiment seems to have been wholly devoted to the deep electromagnetic sounding of the Earth's crust, and no measurements to detect artificially generated Pc 1 pulsations were reported.

We conclude that the proposed 'peninsula method' for ULF signal generation in the ionosphere and magnetosphere¹ is not only feasible but that it is likely to have considerable economic advantages over the use of a large horizontal wire loop on the ground. These advantages include lower construction and running costs and the production of much greater ULF magnetic field amplitudes in the lower ionosphere per unit of input power.

We thank D. M. Bubenik, J. L. Buxton and H. Lewis for assistance, Mr and Mrs G. F. Hodder and the late Mr and Mrs E. Hartell on Chappaquiddick for their co-operation, and also the personnel at the US Naval Air Station at Brunswick, Maine, who made the airborne measurements. This work was supported by the US Defense Advanced Research Projects Agency through an Office of Naval Research Contract and by the Office of Naval Research.

Received 20 February; accepted 9 May 1980.

1. Lipa, B. J., Fraser-Smith, A. C., Villard, O. G. Jr & Storey, J. R. *Nature* **258**, 311–313 (1975).
2. Greifinger, C. *J. geophys. Res.* **77**, 6761–6773 (1972).
3. Harker, K. J. *J. geophys. Res.* **80**, 3100–3110 (1975).
4. Morokhov, I. D., Velikhov, Ye. P. & Volkov, Yu. M. *Atomnaya Energiya* (in Russian) **44**, 213–219 (1978).
5. Gorbunov, G. I. *et al. Dokl. Akad. Nauk SSSR* **247**, 578–582 (1979).

$\Sigma^\pm$ lifetimes and longitudinal acceleration

C. E. Roos, J. Marraffino, S. Reucroft*, J. Waters, M. S. Webster* & E. G. H. Williams

Department of Physics and Astronomy,
Vanderbilt University, Nashville, Tennessee 37235

A. Manz, R. Settles* & G. Wolf

Max-Planck-Institut für Physik und Astrophysik, Föhringer Ring 6,
D-8000 München 40, FRG

The interactions of 420–500 MeV/c K^- in the 11T hydrogen bubble chamber HYBUC have been used to produce a sample of 120,000 $\Sigma^\pm$ decays in flight^{1,2}. We describe here how these decays were used to investigate the effects of longitudinal accelerations between 0.5 and 5.0×10^{15} g on particle lifetimes. Any observed effect of acceleration on lifetime would indicate that the Lorentz transformation and/or the dynamics of particle decay is sensitive to acceleration. These data suggest that accelerations which change the Lorentz factor and which can return a relativistic elementary particle clock to rest do not cause any observable change in lifetime. This null result complements earlier measurements of the effects of transverse acceleration on muons¹³. Thus ensembles of elementary particles are ideal clocks which are invariant under intense accelerations.

The momentum range of the $\Sigma^\pm$ in the HYBUC sample (200–650 MeV/c) is particularly suited to the study of the effects of acceleration on lifetime, because the proper time for decay in the sigma rest frame is a significant fraction of the stopping time as measured in the laboratory. The mean decay length for a momentum of 200 MeV/c at production is 40% of the particle range for Σ^- and 21% for the Σ^+ . The average value of this ratio over the momentum range of this experiment is 10% for the Σ^- sample and 5% for the Σ^+ sample.

The slowing down of the sigmas in hydrogen is an electromagnetic process with forces and accelerations that are large compared with gravity. The average accelerations are of the order of 10^{15} g. The instantaneous accelerations are much greater than these average values and estimates are model dependent. Impact parameter calculations give instantaneous accelerations as high as 10^{22} g.

The values of the lifetime $c\tau$ have been determined using the maximum likelihood method with a lifetime function normalized with a finite interval of proper time for each event. The lower limit of these intervals corresponds to cutoffs which eliminate scanning losses due to short sigma tracks (0.4 cm for Σ^+ , and 0.6 cm for Σ^-). The upper cutoff is the minimum of: (1) the path length at which the sigma would have left the chamber; (2) the path length at which the sigma would stop; (3) a large proper time cutoff ($c\tau = 15$ cm for Σ^+ , 20 cm for Σ^-). To take into account the energy loss in liquid hydrogen, the proper time τ is obtained by integrating the instantaneous momentum over the path length,

$$c\tau = m \int dx/p(x)$$

where x is the distance travelled in the laboratory and the

momentum $p(x)$ is obtained from the range-momentum tables and kinematic fits to the event. Note that this integral assumes the accuracy of special relativity applied to transformations from the laboratory system to the particle rest frame.

As the acceleration which a particle experiences depends on its momentum, we have examined $\Sigma^\pm$ lifetimes as a function of both the momentum at production and decay. Using the momentum at production is less subject to bias than at decay. However, if the history of acceleration has any observable effect on an elementary particle, then one could distinguish particles on the basis of this effect and, therefore, the momentum and rate of energy loss at the time of decay are of interest. Nuclear interactions are momentum dependent and could affect the lifetimes as a function of momentum. They, however, are <1% in our sample and the scanning requirement of a clear decay vertex would exclude small angle scattering events². The $\Sigma^\pm$ samples were divided into five equal momentum bins as functions of the momentum at both production and at decay. The 20 different lifetime determinations were obtained by a maximum-likelihood method and are plotted in Figs 1 and 2. There is no measured variation of lifetime as a function of longitudinal acceleration at the level of a few per cent and ensembles of sigmas behave as ideal clocks, which are not changed by average stopping accelerations of the order of 10^{16} g.

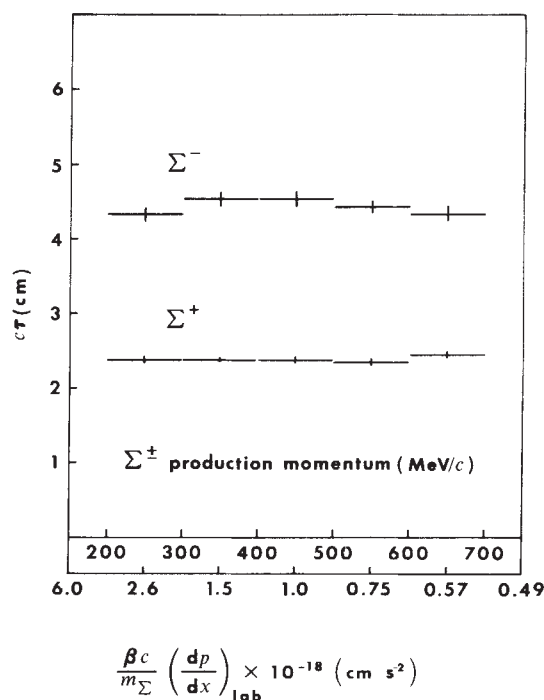


Fig. 1 The $\Sigma^\pm$ lifetimes $c\tau$ (cm) as a function of production momentum and rest frame longitudinal acceleration.

Broadening the discussion we note that our mean values of $c\tau$ for the $\Sigma^\pm$ are in close agreement with earlier experiments³. The particle data tables do not show any observed systematic difference in elementary particle lifetimes as a function of the stopping power of the detector. Sigma lifetimes have been measured in 'free flight'⁴ and in nuclear emulsion⁵⁻¹² and these measurements are also plotted in Fig. 2. These lifetimes are constant to within a few per cent.

The effect of transverse acceleration on the muon lifetime has been investigated by the $g-2$ muon experiment at CERN¹³. Relativistic muons were held in an orbit by magnetic fields which produced accelerations of 10^{18} g perpendicular to the muon's

* Present address: EP Division, CERN, Geneva, Switzerland.

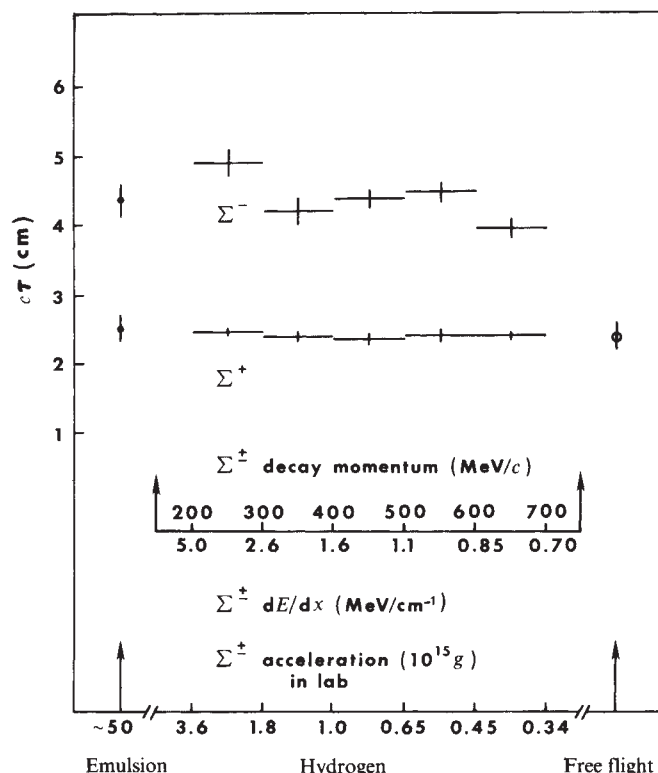


Fig. 2 The $\Sigma^\pm$ lifetimes $c\tau$ (cm) as a function of momentum at decay. The longitudinal acceleration depends on the stopping power of the detector. The lifetimes from HYBUC data (hydrogen) are compared with the lifetimes measured with emulsion⁵⁻¹² and free flight⁴. The rate of energy loss at minimum ionization is 22 times greater in emulsion than hydrogen.

velocity. The measured values of $c\tau$ for orbiting and free muons were the same to better than 1%. This remarkable result indicates that in the case of transverse acceleration, ensembles of muons can be considered as an ideal clock¹³.

Longitudinal acceleration permits us to start two clocks in the laboratory, to accelerate one, return it to rest and then to compare it with the clock that has remained at rest. Transverse accelerations alone would not permit the same experiment. The data indicate that the behaviour of a clock is independent of both longitudinal and transverse accelerations. Arguments that accelerations change the clock are not correct.

We have shown that there is no observed dependence of lifetime on acceleration at the level of a few per cent over the range from 'free flight' to $10^{18}g$, and we conclude that the Lorentz transformation gives the correct relationship between clocks subject to acceleration.

We thank Dr J Bell for enjoyable discussions and critical comments. This work was supported by the NSF grant 073-08392 and the Bundesministerium für Forschung und Technologie, FRG.

Received 4 February; accepted 2 May 1980.

1. Settles, R. *et al.* *Phys. Rev. D* **20**, 2154-2169 (1979).
2. Marraffino, J. *et al.* *Phys. Rev. D* **21**, 2501-2509 (1980).
3. Particle Data Group *Phys. Lett.* **75B**, 1-250 (1978).
4. Cook, V., Ewart, T., Masek, G., Orr, R & Platner, E. *Phys. Rev. Lett.* **17**, 223-228 (1966).
5. Puschel, W. *Nucl. Phys.* **20**, 254-274 (1960).
6. Evans, G. *et al.* *N. Cimento* **15**, 873-898 (1960).
7. Freden, S. *et al.* *N. Cimento* **16**, 611-624 (1960).
8. Kaplon, M. *et al.* *Ann. Phys.* **9**, 139-168 (1960).
9. Chiesa, A. *et al.* *N. Cimento* **19**, 1171-1182 (1961).
10. Barkas, M. *et al.* *Phys. Rev.* **124**, 1209-1223 (1961).
11. Bhowmik, B. *et al.* *Nucl. Phys.* **53**, 22-32 (1964).
12. Tovee, D. *et al.* *Nucl. Phys.* **B33**, 493-514 (1971).
13. Bailey, J. *et al.* *Nature* **268**, 301-304 (1977).

Time-temperature relations in the magnetization of assemblies of single domain grains

D. Walton*

Department of Physics, McMaster University, Hamilton, Ontario, Canada L8S 4M1

A theory for important magnetization processes such as magnetic viscosity and the production of a moment on cooling in a magnetic field is presented here. The results for viscous phenomena differ from those obtained by others in that the time dependence of the viscosity depends on the size distribution of the magnetic grains. The moment produced on cooling in a magnetic field is calculated for an arbitrary distribution of grains for the first time. The effect of the cooling rate on the moment produced is found not only to be independent of that distribution but also of the temperature dependence of the saturation magnetization.

The moment produced on cooling is affected by the rate of cooling. Cooling rate effects have been considered by York¹, Pullaiah *et al.*² and Dodson and McClelland-Brown³ who all derive results for assemblies of identical grains. They show that the blocking temperature is higher, the higher the cooling rate, and the resultant moment is reduced. In determining palaeointensities, then, a difference in cooling rates will lead to differences in the field intensity deduced. When rocks which are cooled over millions of years are measured using cooling times of minutes the difference can be quite large, and a severe overestimate of the ancient field will result.

Previous authors have only calculated this effect for assemblies of identical grains. However, the magnetization which is observed is an integral over a distribution of grains of different sizes. This integral is found not to be sensitive to the grain size distribution, and the cooling rate effect to be sample dependent only if the magnetic constituents are different.

The calculation of magnetic viscosity presented here uses fewer approximations than generally used, and the time-temperature relation obtained differs from the expressions which can be found in the literature (see ref. 4 for detailed references).

All materials which display viscosity are in disequilibrium. The viscosity is the evolution of the system towards an equilibrium state. Consider an assembly of identical particles, of which n^+ are oriented so that their energy is higher than that of the remaining n^- which are in a ground state. The difference $n = n^+ - n^-$ will tend towards an equilibrium value n_{eq} at a rate given by

$$\frac{dn}{dt} = -\frac{(n - n_{eq})}{\tau} \quad (1)$$

where τ is a relaxation time.

This equation integrates easily to yield

$$n - n_{eq} = (n - n_{eq})_{t=0} e^{-t/\tau} \quad (2)$$

Note that although this calculation is for single domain grains, the results apply to all systems which obey equation (1).

If equation (2) is multiplied by the appropriate moment and integrated over all assemblies the change in total magnetization with time is obtained. The moment is JV where J is the saturation magnetization per unit volume.

The relaxation time is given by

$$\tau^{-1} = c e^{-KV/kT} \quad (3)$$

* Present address: Department of Geodesy and Geophysics, University of Cambridge, Madingley Road, Cambridge CB3 0EZ, UK.

Where c is a rate assumed to be constant, K is the anisotropy constant, k is Boltzmann's constant and T the temperature. Only K and V will vary. Thus defining $W_V(K)$ as the probability that a grain of volume V will have a K lying between K and $K + dK$, similarly that $N(V)$ is the probability that V lies between V and $V + dV$, and N is the number of grains

$$M(t) = M_{eq} + N \int \int W_V(K) JV(n - n_{eq})_{t=0} \times \exp[-ct e^{-KV/kT}] N(V) dV dK \quad (4)$$

where

$$M_{eq} = N \int \int n_{eq} JV N(V) W_V(K) dV dK.$$

The first term in the integrand corresponds to viscous decay of the initial magnetization in zero field, the second to the acquisition of a moment from a demagnetized state.

Evaluating the time derivative of equation (4) and assuming that $W(K)$ is independent of V :

$$M'(t) = N \int W(K) dK \int JV \Delta n_0(K, V) [-c e^{-KV/kT}] \times \exp[-ct e^{-KV/kT}] N(V) dV \quad (5)$$

where $\Delta n_0(K, V) = (n - n_{eq})_{t=0}$.

As $c = 10^{10} \text{ s}^{-1}$ the integrand is small for small KV . It increases rapidly with KV as KV approaches about 30 kT, reaches a maximum, and decays exponentially as KV increases further due to the term $e^{-KV/kT}$.

Thus the V integration can be performed using saddle point methods⁵. If V_B is the solution of $KV_B = kT \ln ct$

$$tM'(t) = \int_{K_{min}}^{K_{max}} \frac{(2\pi)^{1/2}}{e} \Delta n_0(K, V_B) N(V_B) \times \frac{kT}{K} JV_B W(K) dK \quad (6)$$

A suitable average, $\bar{K}$, can always be found such that

$$tM'(t) = \frac{(2\pi)^{1/2}}{e} \Delta n_0\left(\bar{K}, \frac{kT}{\bar{K}} \ln ct\right) N\left(\frac{kT}{\bar{K}} \ln ct\right) \times \left(\frac{kT}{\bar{K}}\right)^2 (\ln ct) J \quad (7)$$

$\bar{K}$, of course, is temperature dependent, and must lie between the maximum and minimum possible values of K .

This result leads to three conclusions: first, that the time and temperature dependence are connected through $T/\bar{K}$ and $\ln ct$. Second, that the form of this relationship depends on the previous history of the sample, and it is impossible to draw general conclusions from experimental results unless the initial state has been specified. Third, that it depends on the grain size distribution. This last result differs from Dunlop's⁴ who concludes that this distribution is not critically important.

The simplest initial state is probably that in which the sample has been completely demagnetized. Consider then the acquisition of a viscous moment by a completely demagnetized sample:

In this case

$$\Delta n_0 = -n_{eq} = -JV_B H / 3kT \quad (8)$$

and

$$tM'(t) = \frac{(2\pi)^{1/2}}{3e} \frac{J^2 H}{\bar{K}} \left(\frac{kT}{\bar{K}} \ln ct\right)^2 N\left(\frac{kT}{\bar{K}} \ln ct\right) \quad (9)$$

It should be possible to express the distribution as a power series

$$N(V) = \sum_r a_r V^r$$

In the following only one term in the series will be used, but the extension to the general case is obvious.

Thus, assuming $N(V) = AV^r$, and if the anisotropy is due to the shape of the grains, $\bar{K} = aJ^2$, and

$$tM'(t) = BH \left(\frac{kT \ln ct}{J^2}\right)^{2+r} \quad (10)$$

where B is a constant.

A parameter which is frequently quoted is the viscosity coefficient.

$$\int = \frac{\partial M(t)}{\partial \ln t} = tM'(t) \quad (11)$$

S is often found to be proportional to the temperature^{3,4,6}. This is true at low temperatures where J does not change very much, therefore r must be -1 . (Note that a log normal distribution yields $r \sim -1$ for the larger grains.) However, equation (10) would suggest that the intervention of the temperature dependence of J would lead to a more complex temperature dependence in general.

Equation (10) is readily integrated to yield

$$M(t) = \frac{BH}{3+r} \left(\frac{kT}{J^2}\right)^{2+r} (\ln ct)^{3+r} \quad (12)$$

A good way to determine $N(V)$ or r would be to plot $M(t)$ versus T (or T/J^2 if high temperature results are used) for equal values of t .

The expression obtained for the viscous moment differs from those obtained by others.

First, the moment depends on $(\ln ct)^m$ where m is determined by the grain size distribution. In general, however, it will be difficult to observe this experimentally because $(\ln ct)^m \approx (\ln c)^m + (\ln c)^{m-1}(\ln t)$, and a $\ln t$ dependence will be observed except after very long times.

Second, the simple relationship $M(T_1 \ln ct_1) = M(T_2 \ln ct_2)$ which is frequently used⁷ will not hold in general.

The time and temperature dependence of magnetic viscosity depends on the previous history of the sample. Therefore, unless $N(V)$ at $t = 0$ is known an experiment on magnetic viscosity may yield bizarre, but meaningless, results. This is probably most important for experiments on viscous decay. On the other hand, if viscous acquisition is being measured it is probably safest to start with a completely demagnetized sample.

Finally, an interesting comparison of equation (12) with experiment is possible: Pullaiah *et al.*³ remark that a magnetization acquired by heating a specimen of magnetite at 400 °C for 10 days is not removed before 550 °C if heating steps of 5 min are used. The samples were, in fact, expected to be completely demagnetized by 450 °C if the relationship between time and temperature is the one they derive, namely

$$\frac{T_1 \ln ct_1}{[J(T_1)]^2} = \frac{T_2 \ln ct_2}{[J(T_2)]^2}$$

However, equation (12) indicates that the relation should be

$$\left(\frac{T_1 J^2(T_2)}{T_2 J^2(T_1)}\right)^{2+r} = \left(\frac{\ln ct_1}{\ln ct_2}\right)^{3+r}$$

With this result if $r = -1$ (which roughly corresponds to a log normal distribution), the temperature for complete demagnetization becomes 500 °C, and for $r = -1.6$ it is 550 °C.

Similar arguments can be used to explain Pullaiah *et al.*'s observation that unblocking of NRM occurs at temperatures lower than they expected.

Next it is interesting to calculate the moment acquired during cooling.

The solution of equation (1) is complicated by the fact that n_{eq} and T are functions of time. Formally the solution is

$$n = \left\{ \exp\left(-\int_0^{t_i} \frac{dt}{\tau}\right) \right\} \int_0^{t_i} \frac{n_{eq}(t)}{\tau} \exp\left[+\int_0^t \frac{dt}{\tau}\right] dt \quad (13)$$

where t_i is the time at which the moment is measured after the

material has cooled. Equation (13) may be rewritten

$$n = \int_0^{t_i} \tau^{-1} n_{eq}(t) \left\{ \exp \left[- \int_t^{t_i} \tau^{-1} dt \right] \right\} dt \quad (14)$$

For small t , that is initially, the argument of the exponential is large and therefore the integrand is small. It is small again as $t \rightarrow t_i$ because the relaxation time τ becomes very large. Thus, the method of steepest descents is again appropriate, and letting

$$g(t) = -\frac{KV}{kT} - \int_t^{t_i} c e^{-KV/kT} dt \quad (15)$$

the value of n is

$$n = (2\pi)^{1/2} \frac{n_{eq}(t_c)c}{[g''(t_c)]^{1/2}} \exp \left[-\frac{KV}{kT(t_c)} - \int_{t_c}^{t_i} \frac{dt}{\tau} \right] \quad (16)$$

t_c is obtained from $g'(t_c) = 0$. In fact t_c will never be needed explicitly, but the condition $g'(t_c) = 0$ gives, if $d/dt(K/T) = \alpha$

$$\frac{KV}{kT} = \ln \left(\frac{kc}{V\alpha} \right) \quad (17)$$

This equation may be solved for the temperature; this temperature would then be the blocking temperature. It will be convenient to modify the definition slightly so that the blocking temperature is

$$T_B = \frac{(2\pi)^{1/2}}{e} \frac{K_B V}{k \ln(kc/V\alpha)} \quad (18)$$

where K_B must be evaluated at the blocking temperature.

Now, returning to equation (16) we can evaluate n by introducing the expression for n_{eq} , equation (8)

and

$$g''(t_c) = -\frac{V}{k} \frac{d\alpha}{dt} - \left(\frac{V\alpha}{k} \right)^2 \quad (19)$$

In equation (16) the values of J and α , which are temperature-dependent, are evaluated at T_B . For single domain grains, where shape anisotropy dominates, K is proportional to J^2 (ref. 8).

The parameter α depends on the particular way in which the material cools and is temperature and time dependent in general. The algebra becomes simpler if α is assumed to be constant. This is a relatively good approximation: it corresponds to a cooling law such that $d/dt(1/T) = \text{constant}$. A similar approximation is made in ref. 3. If α is constant

$$\int_{t_c}^{t_b} \tau^{-1} dt = -\frac{k}{\alpha V} \left[\left(\frac{1}{\tau} \right)_{t=t_b} - \left(\frac{1}{\tau} \right)_{t=t_c} \right] = 1 \quad (20)$$

as

$$(\tau)_{t=t_b} \gg (\tau)_{t=t_c} = \tau_b$$

combined with equations (20) and (21), (16) yields the eminently reasonable result that n is given by the equilibrium expression evaluated at the blocking temperature:

$$n = \frac{J_B V H}{3kT_B}$$

The magnetization is now

$$M = N \int \int J V \left[\frac{J_B V H}{3kT_B} \right] N(V) W(K) dV dK \quad (21)$$

K may be replaced by its average as before, however, its temperature dependence must be retained.

Assuming that $N(V)$ obeys a power law $N(V) = AV^r$, equation (21) becomes

$$M = \frac{NJ_B H A}{3k} \int_{V_0}^{V_m} \frac{J_B}{T_B} V^{2+r} dV \quad (22)$$

It is convenient to integrate with respect to either J_B or T_B .

Assuming that $\bar{K} = bJ^p$ and that $J = J_0(1 - T/T_c)^{1/n}$, equation (22) yields a hypergeometric function which reduces to

$$M \equiv \frac{NAJH}{3kT_c} \frac{p}{n} (V_m)^{3+r} \left[\left(\frac{kT_c}{V_m b J_0^p} \right) \ln \frac{ke}{\alpha V_{eff}} \right]^{1/p} \quad (23)$$

In obtaining this result $\ln kc/V\alpha$ which varies slowly with V has been assumed to be constant, and taken outside the integral. V_{eff} is an appropriate average V .

V_m is the blocking volume at the highest temperature of interest. Most of the moment is produced in the range from about 50 K below the Curie temperature to room temperature. Therefore V_m will be taken to be the volume of those grains whose blocking temperature is ~ 50 K below the Curie temperature. V_m is ~ 30 times V_0 , the blocking volume at room temperature.

Note that the grain size distribution only influences the cooling rate effect by possibly affecting V_{eff} , and this is completely negligible. Note also that the cooling rate dependence does not depend on the temperature dependence of J , at least of the order of the approximation used here.

We can now estimate the effect of a change in cooling rate on the resultant moment: $\ln(kc/V_{eff}\alpha)$ may be estimated as follows, $\alpha = [\Delta(\bar{K}/T)/\Delta t]$ so that in going from the Curie temperature to room temperature $\bar{K}/T$ increases from zero to K_0/T_0 where K_0 and T_0 refer to room temperature, thus

$$\frac{kc}{V_{eff}\alpha} = \frac{kT_0}{V_{eff}K_0} c \Delta t$$

now $V_{eff}K_0/kT_0$ is of the order of 30 and if $c \sim 3 \times 10^{10}$

$$\ln \left(\frac{kc}{V_{eff}\alpha} \right) \cong \ln(10^9 \Delta t)$$

If Δt is changed by a factor of 10 the moment should change by about 6% for magnetite. For haematite⁴, on the other hand, if $K = aJ^4$ the change should only be about 3%.

These results are in broad agreement with the calculations of Dodson and McClelland-Brown³, in the sense that the integrated change in moment is in the low range of values obtained by them for an assembly of identical grains.

At present the only experimental results are those of Fox and Aitken⁹: for various samples of pottery they obtain an increase of between 3% and 9% in the moment for a decrease of an order of magnitude in the cooling rate. This agrees with the results obtained above as the carriers of the magnetization in pottery are believed to be monodomain grains of magnetite. It would be desirable, however, to have more precise experimental results.

An approximate expression for the moment produced during cooling from just below the Curie temperature has been obtained. We conclude that: the effect of cooling rate on the resulting moment is not sensitive to the grain size distribution or the temperature dependence of J . But is sensitive to the form of the temperature dependence of K on J ; an order of magnitude increase in cooling rate leads to a 6% decrease in moment for magnetite, but only 3% for haematite.

I thank Dr D. J. Dunlop for comments, Dr M. J. Aitken for communicating experimental results before publication. This work was supported by a grant from the Natural Science and Engineering Council of Canada.

Received 5 February; accepted 23 May 1980.

1. York, D. *Earth planet. Sci. Lett.* **39**, 94-97 (1978).
2. Pullaiah, G., Irving, E., Buchan, K. L. & Dunlop, D. J. *Earth planet. Sci. Lett.* **28**, 133-145 (1975).
3. Dodson, M. H. & McClelland-Brown, E. J. *geophys. Res.* (in the press).
4. Dunlop, D. J. *Rev. Geophys. Space Phys.* **13**, 855-901 (1973).
5. Arfken, G. *Mathematical Methods for Physicists*, 2nd edn (Academic, New York, 1970).
6. Shimizu, Y. J. *Geomagn. Geoelectr.* **11**, 125-138 (1960).
7. McElhinny, M. W. *Paleomagnetism and Plate Tectonics* (Cambridge University Press, 1973).
8. Stacey, F. D. & Banerjee, S. K. *The Physical Principles of Rock Magnetism* (Elsevier, Amsterdam, 1974).
9. Fox, J. M. W. & Aitken, M. J. *Nature* **283**, 462 (1980).

Identification and estimation of carbonate minerals at low levels by evolved CO₂ analysis

A. E. Milodowski

Department of Geology, King's College, Strand,
London WC2R 2LS, UK

D. J. Morgan

Institute of Geological Sciences, 64/78 Gray's Inn Road,
London WC1X 8NG, UK

Recent differential thermal analysis (DTA) investigations of carbonate minerals using a flowing CO₂ furnace atmosphere^{1,2} have shown that amounts as low as 0.25% (2,500 p.p.m.) may be detected in natural or synthetic mixtures, a significant improvement on that possible using static air DTA or conventional X-ray diffraction techniques. We show here that carbonate minerals can be routinely detected in the hundreds of p.p.m. concentration range using a non-dispersive IR CO₂ detector linked to a DTA furnace and describe modifications to the technique which permit the detection of certain carbonates below 100 p.p.m. The method described gives both an analytical

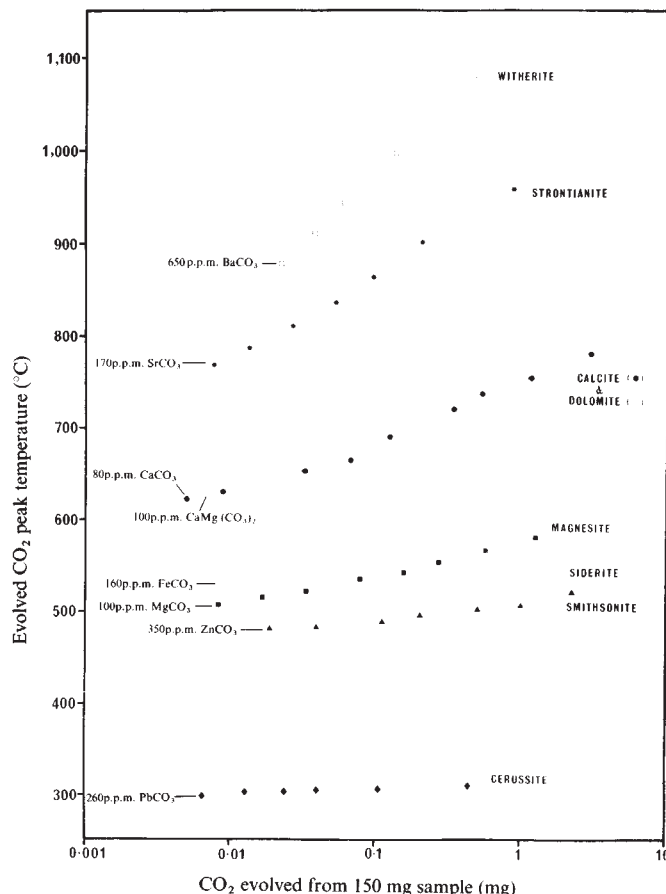


Fig. 2 Plot of evolved CO₂ peak temperature versus log mass of CO₂ evolved for the eight carbonates investigated.

determination of CO₂ and an identification of the mineral species responsible for CO₂ generation. It should be of use in the study of trace constituents in rocks, sediments, soils and ores and as a quality control technique in industrial processes.

Details of the DTA-EGA (CO₂) system have been given previously³. Briefly, this consists of a DTA furnace swept at 300 ml min⁻¹ by a carrier gas stream consisting of a 2:1 mixture by volume of nitrogen and oxygen and linked by a stainless steel line to a non-dispersive IR CO₂ detector. This has two analysis cells capable of measuring up to 1,000 and 10,000 p.p.m. CO₂ in the carrier gas stream, although for the present study only the most sensitive cell was used. Output signals from the detector are fed into an external chart recorder which also monitors sample temperature. Additional, more sensitive ranges on the chart (0–500 and 0–100 p.p.m. fsd) are obtained through ×2 and ×10 signal amplification switches on the detector.

A standard mass of sample (150 mg) is lightly packed into a dimpled Pt-crucible and this placed in the DTA head. A heating rate of 15 °C min⁻¹ is used. Areas of the CO₂ evolution profiles on the chart are measured with a planimeter and the amount of CO₂ evolved obtained by calculation³.

Eight X-ray pure natural anhydrous carbonates were examined: witherite, strontianite, calcite, dolomite, magnesite, siderite, smithsonite and cerussite. Each mineral was ground to pass a 200-mesh sieve and mixed with alumina to give a dilution sequence covering the range 2% to 50 p.p.m. The alumina/carbonate mixtures were placed in glass screw-top bottles and mechanically shaken for 20 min. No precautions were taken to obtain a statistically uniform 150 mg sample for analysis as the amount of carbonate mineral present was determined from the area of the CO₂ evolution profile.

Figure 1a–c shows CO₂ evolution profiles given by the eight carbonates at nominal concentrations ranging from 0.2% to 50 p.p.m. At 0.2% (Fig. 1a) all the carbonates give sharp peaks

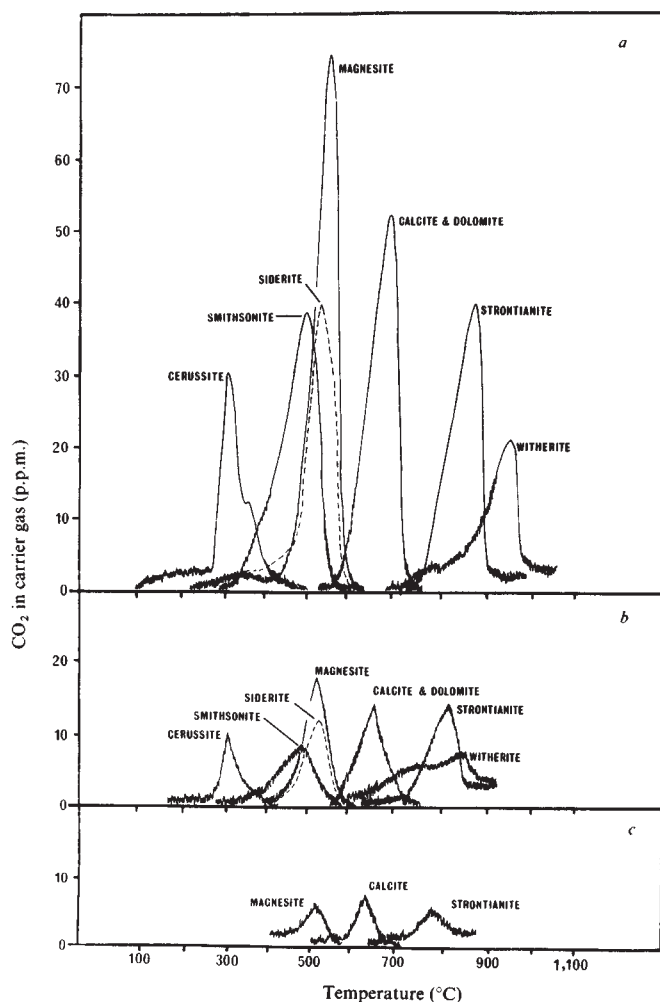


Fig. 1 CO₂ evolution profiles obtained from: a, 0.2%; b, 500 p.p.m.; and c, 50 p.p.m. nominal concentrations of carbonate minerals in alumina. Evolution profiles in a and b were obtained using a carrier gas flow rate of 300 ml min⁻¹ and in c with a flow rate of 100 ml min⁻¹.

with virtually no baseline drift. Areas of the peaks vary in proportion to the CO₂ contents of the minerals which range from 52.2% for magnesite to 16.5% for cerussite. Detection limits are obviously influenced by this factor, although with decreasing concentrations other factors become increasingly important. In the concentration range examined, CO₂ release from witherite is governed more by solid-state reaction with alumina than by simple dissociation⁴. As a result, CO₂ evolution occurs over a relatively large temperature range and the mineral does not give recognizable peaks at concentrations <500 p.p.m. Kinetics of the dissociation are also relevant: cerussite, although containing less than half the CO₂ content of smithsonite, can be detected at lower levels because of its sharp CO₂ evolution profile (Fig. 1a, b).

Figure 2 summarizes the data obtained for the dilution sequences using a carrier gas flow rate of 300 ml min⁻¹. Values in p.p.m. to the left of the figure represent the lowest concentration at which each mineral gives a well-defined CO₂ peak. Reducing the carrier gas flow rate would be expected to improve detection limits due to the effective increase in CO₂ content of the carrier gas. However, at slower flow rates convection currents in the furnace assume a much greater importance. These are especially noticeable in the 150–450 °C range and cause irregular flushing of the evolved CO₂ and a consequent increase in background noise-to-signal ratio in the detector. Detection limits for cerussite and smithsonite, which dissociate within this temperature range, were not, therefore, significantly improved at slower carrier gas flow rates although satisfactory CO₂ peaks were obtained from magnesite, calcite and strontianite at nominal concentrations of 50 p.p.m. using a carrier gas flow rate of 100 ml min⁻¹ (Fig. 1c).

Plots of evolved CO₂ peak temperature versus log mass of CO₂ evolved (Fig. 2) are straight lines which become shallow curves in the lower concentration ranges (this curvature is experimental and is a function of the rate of diffusion of CO₂ from the carbonate–alumina mixture). In general, the higher the dissociation temperature of the carbonate, the greater the slope of the line, a similar relationship to that existing between plots of DTA dissociation peak temperature and mass of the various carbonates at higher concentrations⁵. The exception is siderite which shows no variation in CO₂ peak temperature with dilution; note that Warne⁶ found that the temperature of the endothermic DTA peak of this mineral was also relatively insensitive to dilution.

The CO₂ peak temperature–concentration relationships presented in Fig. 2 provide the basis for both identification and quantification of individual carbonates present at low levels in natural mixtures although it is apparent that distinction between calcite and dolomite is impossible in the experimental conditions used and some difficulty might be anticipated in differentiating between magnesite, siderite and smithsonite, especially when variations in thermal behaviour of the natural minerals are taken into account. This topic will be discussed in detail elsewhere but note here that placing a porous cap over the Pt-crucible resulted in a marked separation of dolomite and calcite peaks at concentrations of 1% and above (Fig. 3). Evolution of CO₂ from magnesite is also retarded to a much greater extent than from siderite or smithsonite by the porous cap and this mineral is readily distinguishable in 0.5% artificial mixtures with the other two carbonates.

This work was carried out while A.E.M. was in receipt of a Natural Environment Research Council CASE studentship. The paper is published by permission of the Director, Institute of Geological Sciences.

Received 10 March; accepted 6 May 1980.

1. Warne, S. St. J. *Nature* **269**, 678 (1977).
2. Warne, S. St. J. & Mitchell, B. D. *J. Soil Sci.* **30**, 111–116 (1979).
3. Morgan, D. J. *J. Thermal Analysis* **12**, 245–263 (1977).
4. Wentworth, S. A., Sharp, J. H. & Bye, G. C. *Proc. Soc. analyt. Chem.* **5**, 245 (1968).
5. Smykatz-Kloss, W. *Differential Thermal Analysis* (Springer, Berlin 1974).
6. Warne, S. St. J. *Chemie Erde* **35**, 251–255 (1976).

Coral morphology, diversity and reef growth

John Chappell

Department of Biogeography and Geomorphology, Australian National University, Canberra, Australia

The shape of reef corals is affected by light level^{1,2} and by wave stress^{3,4}, leading to the well-known zonation of coral form associations with exposure and depth^{3–6}. Growth form also seems to be constrained by geometric factors^{2,7,8}. Similarly, coral calcification rate, of importance both for coral form and for net reef growth, is light dependant^{9,10}, and may be affected by other factors such as wave stress and sediment flux. Here the growth structure of raised Pleistocene reefs in New Guinea, of both fringing and barrier types, is explained in terms of variations of coral framework growth rate across the reef profile. A model for growth rate in terms of wave stress, light level and other variables is outlined, first by examining the effects of these factors on coral form and diversity.

Growth form responses to several factors are outlined in Fig. 1, on the following basis. (1) Light response: Porter's general argument that surface-to-volume ratio should diminish as light increases⁷ is contradicted by types which show globose to platy polymorphism^{1,2}. A better index, consistent with Porter's argument, is the ratio of polyp-covered surface to the projected surface area seen from above. (2) Hydrodynamic stress response: adaptation will be to increase structural strength as wave/current stress increases^{3,11,12}. (3) Sediment flux effect: advantageous forms, where sediment flux is high, would intercept the smallest proportion of settling sediment per unit surface area, for a given polyp size¹³. (4) Subaerial exposure: significant on many reef flats, the best adaptation is likely to be that which maximizes stored water capacity by minimizing surface-to-volume ratio as well as maximizing sideways spreading. Note that Fig. 1, outlining these responses, is not exhaustive and interpolations can be made in any row, for example, types 7 and 8 have similar or lower polyp surface/projected surface ratios than type 1, and can be placed at the left in row 1.

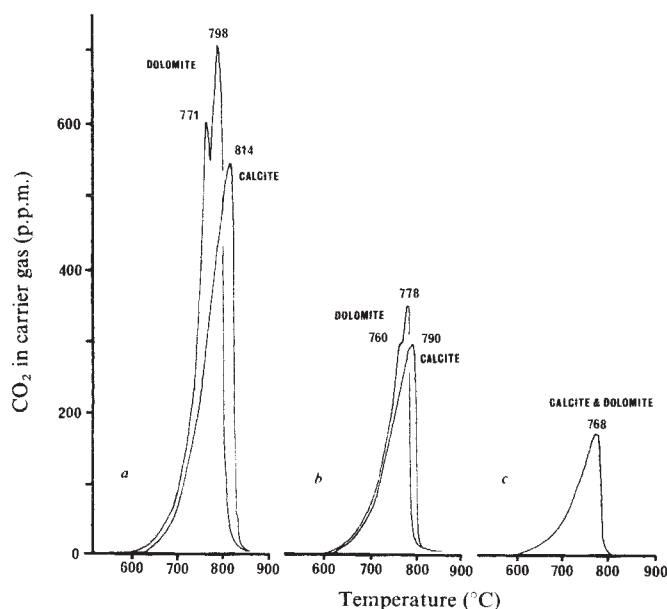


Fig. 3 CO₂ evolution profiles of calcite and dolomite obtained with a porous cap on top of sample crucible. a, 2%; b, 1%; and c, 0.5% nominal concentrations.

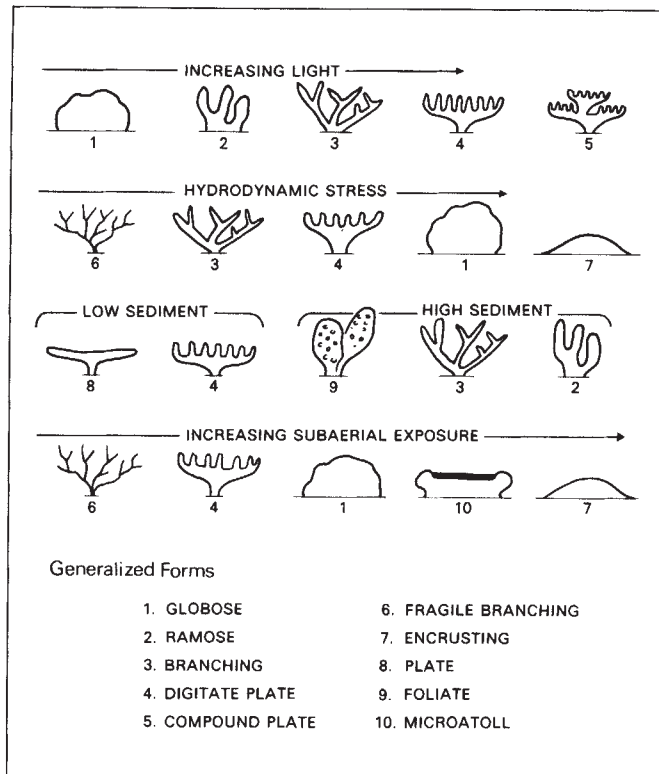


Fig. 1 Coral form responses to environmental stresses.

Net reef growth not only depends on coral calcification rate, but is also affected by the type of framework built by the local assemblage of forms. Figure 1 indicates that form assemblage will vary with the net balance of the four stress factors, which vary with position, as follows. Light diminishes with depth,

although it seems not to be stressful until the depth where photo-adaptation (increasing zooxanthellae) ceases to change^{1,14}. Wave stress increases with square of wave height and declines negative-exponentially with depth. The stress imposed by settling sediment should increase slowly down the windward forereef; it is significant on the reef flat, and also is high where sediment overspill to the backreef is high. Subaerial exposure on the reef flat increases with height above lowest spring tide level. These variations are shown in Fig. 2 for an idealized reef cross-section. Characteristic coral community forms, projected in Fig. 2 in the light of Fig. 1, are consistent with zonations described by others^{3,6,15}. Note that when wave exposure is very high, the encrusting coral zone at the reef margin (shown at '7' in Fig. 2) often is replaced by algal bioherm^{3,16}.

Hypothetical diversity curves also are shown in Fig. 2, based on net stress (= sum at any point of stresses shown graphically in Fig. 2). Diversity ranges from maximum when environmental stress is small, down to zero when any factor is limiting¹⁷. Diversity curves in Fig. 2 are based on an assumed inverse-linear relationship between net stress and diversity. The curve for the forereef compares well with Porter's results from a Panama reef¹⁸ when these are smoothed to eliminate variations related to local topographic effects. No account is taken, in compiling Fig. 2, of the 'intermediate disturbance' effect¹⁹ on diversity of events such as cyclones.

Coral morphology and diversity zonation have been accounted for by others in terms of depth variation of wave stress and light^{3,4,16,17}. In this regard, the semi-quantitative relationships underlying Fig. 2 are valuable. It is also suggested that growth of coral framework is constrained by the four stress factors in the same way as is diversity in Fig. 2, that is, that growth diminishes as net stress increases until any factor becomes limiting. This is tested by simulating reef growth and comparing the results with known reef growth structures.

A simple computer model to simulate reef development was arranged as follows. Each of the four stress factors was given a range 0-1 and net stress taken as the arithmetic mean at any point on the evolving profile. Growth ceases where any factor

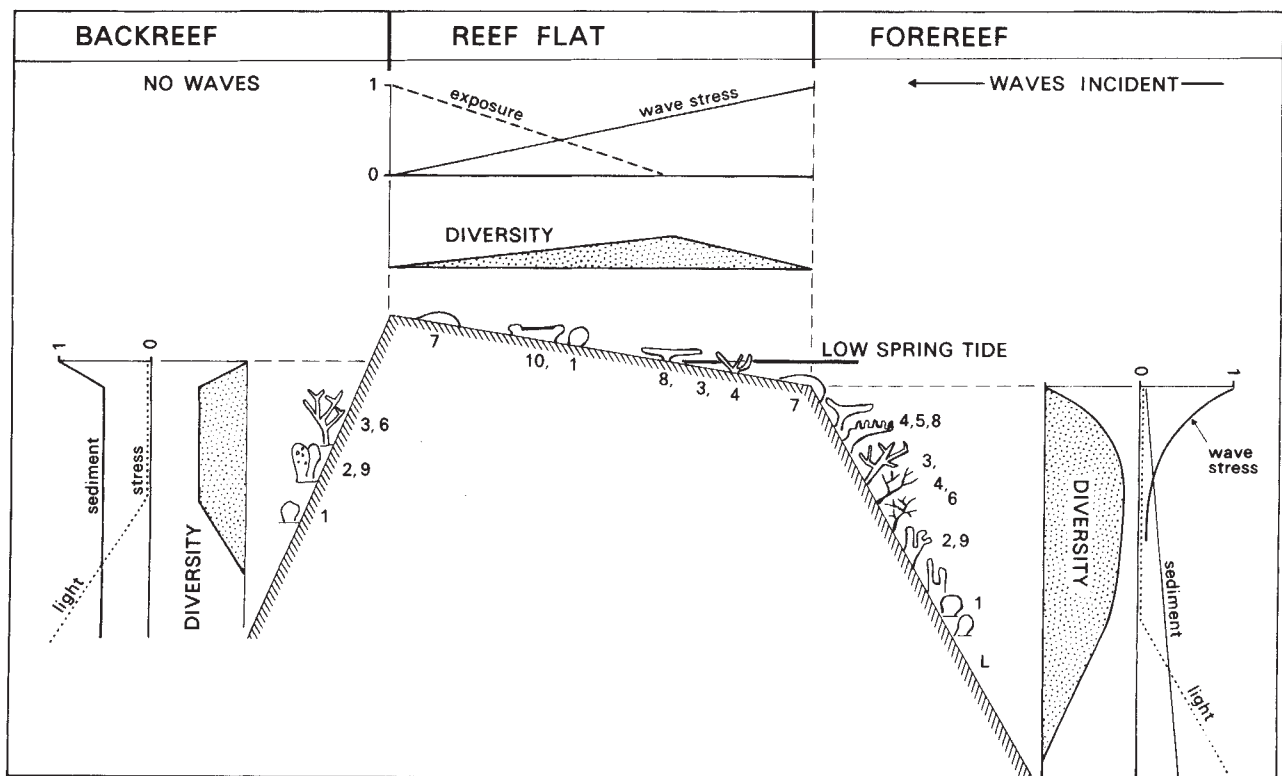


Fig. 2 Idealized variation of coral community forms and diversity from sums of stresses across simple reef section.

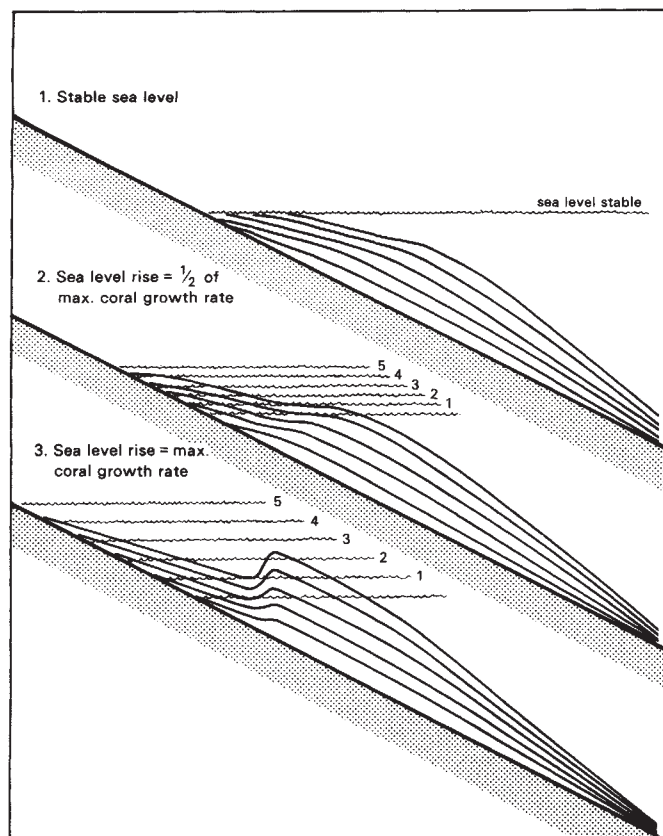


Fig. 3 Reef growth: computer model results using stress factors as in Fig. 2, with different rates of sea-level rise.

reaches the limiting value of 1. Wave behaviour was modelled as Airy waves outside the breaker line, solitary waves inside it, with standard equations for bed shear, and so on.²⁰ Energy dissipation at wave break was assumed to be 75%. Maximum possible growth rate was 1 cm yr^{-1} . Light stress was assumed 0–15 m depth and to increase then to become limiting at 40 m. Waves of 2 m at 8 s (800 W m^{-1} of wave crest) were input (assumed not to be limiting). Sediment flux, taken as equivalent to the rate of wave energy loss from bed friction, was assumed limiting when wave power loss reached 100 W m^{-1} per m of wave advance. These values are taken in accordance with my observations of modern and Pleistocene reefs at Huon Peninsula, New Guinea²¹, with which model results are compared, below. Variations from these values alters scaling dimensions but not the form of the results.

Reef development is affected by previous topography which developed sub-aerially during Pleistocene low sea levels^{22,23}. This is simplified in the model by using a rectilinear initial substrate, which accords with the New Guinea case²¹. The rate of sea-level rise during deglaciation, relative to maximum potential growth rate of framework, is also important for reef growth structure²¹, although the extent to which Darwin's hypothesis, that barrier and lagoon structure arises from rising relative sea level (submergence), is debated²². Accordingly, Fig. 3 shows three runs of the computer model, with sea-level rise rates of 0.0 cm yr^{-1} , 0.5 cm yr^{-1} , and 1.0 cm yr^{-1} respectively. The first two cases induce formation of a fringing reef; in the third case a barrier and lagoon develops.

The model is compared with late Pleistocene reefs for which the growth history is known, shown in Fig. 4. These developed during sea-level changes centred on the last interglacial period, 135–120 kyr ago, and are now exposed well above sea level through tectonic uplift, at Huon Peninsula, New Guinea²¹ (examples 1, 2 in Fig. 4), and at Atauro Island, Timor²⁴ (example 3). Also shown is the pattern of 'absolute' sea-level change

for the period^{24,25}. Mean growth rates differ between the Huon and Atauro examples; Huon reef VIIa grew 50 m in $<10,000 \text{ yr}$ ($>0.5 \text{ cm yr}^{-1}$), while the Atauro reef grew 40 m throughout the entire interval of 145–115 kyr (0.13 cm yr^{-1}). The rate difference is not due to uplift rate or substrate slope differences as these are matched in one or the other of the Huon cases (Fig. 4), and is probably caused by clastic sediment flux which is much greater at volcanic Atauro than at Huon Peninsula, where the hinterland is limestone. This is supported by the fact that Huon Peninsula coasts away from the limestone region have a volcanogenic hinterland, high clastic flux, and only minor reefs. Coral zonation in the Huon reefs, described by Chappell²¹, is consistent with Fig. 2 (no major algal bioherm occurs at reef crest on this moderate-energy coast); these zones are simplified to backreef (B), shallow forereef (S), and so on, in Fig. 4. Major facies boundaries trace the course of reef growth.

The structures in Fig. 4 compare well with computer model predictions in Fig. 3 when growth rate–sea-level rise rate relationships are considered. Maximum growth rates in Holocene reefs at Huon Peninsula, which are comparable to the raised Pleistocene reefs, are around 0.8 cm yr^{-1} (ref. 26). Sea-level rise for the NW Huon section slightly exceeded this, at $\sim 1.0 \text{ cm yr}^{-1}$ (uplift for this section being only 0.05 cm yr^{-1}), and a barrier-lagoon structure developed (see model case 3 in Fig. 3). Relative sea-level rise rate for the south-east Huon case is lower than maximum potential growth rate, at 0.6 cm yr^{-1} , due to rapid tectonic uplift (0.4 cm yr^{-1} at this section), and a fringing reef formed (see model case 2 in Fig. 3). In the Atauro case the potential reef growth rate seems to have been much lower than

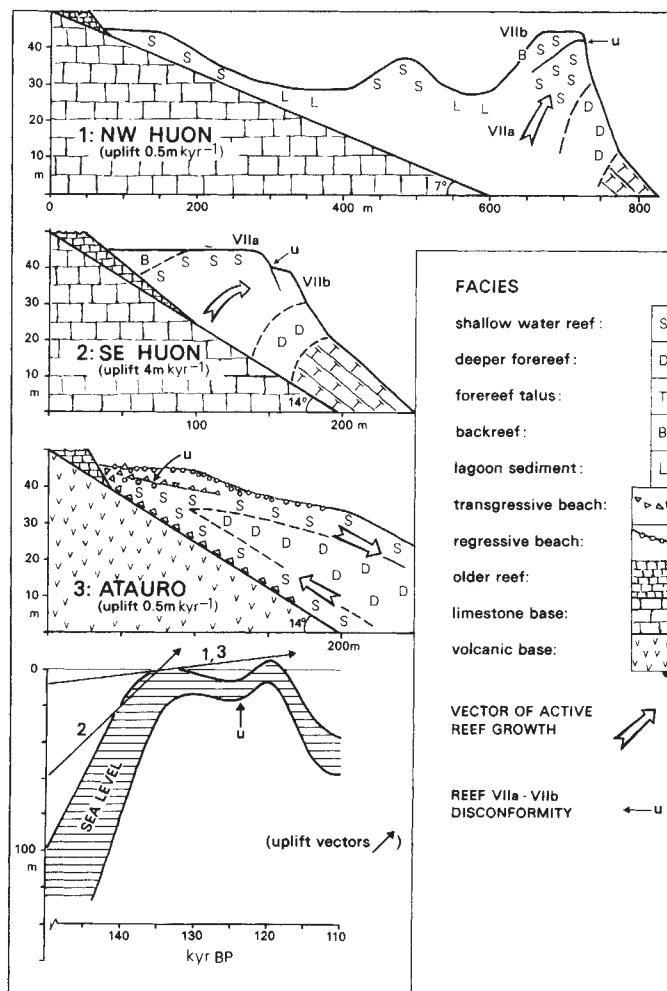


Fig. 4 Mapped cross-sections of late Pleistocene reefs at Huon Peninsula, New Guinea²¹, and Atauro Island, Timor²⁴.

relative sea-level rise rate, resulting in formation of a transgressive shelving reef which continued to grow slowly through the interglacial and into the early phase of the subsequent falling sea level.

In conclusion, raised Pleistocene reefs at Huon Peninsula, where major variations of uplift rate along the coast provide the opportunity for examining reefs formed under different relative rates of sea-level rise, are compatible with a model for reef development which relates growth rate to net environmental stress. As the model is semi-quantitative, its predictions as to growth rates, coral forms and coral diversity, as functions of the four stress factors invoked, are readily testable through further observations.

Received 18 January; accepted 9 May 1980.

1. Jaubert, J. *Proc. 3rd int. Coral Reef Symp. Miami*, **2**, 483–488 (1977).
2. Graus, R. R. & MacIntyre, I. G. *Science* **193**, 895–897 (1976).
3. Geister, J. *Proc. 3rd int. Coral Reef Symp.*, **1**, 23–29 (1977).
4. Morton, J. *Proc. 2nd int. Coral Reef Symp.*, **2**, 55–69 (1974).
5. Vaughan, T. W. *U.S. natn. Mus. Bull.* **49**, 1–427 (1907).
6. Wells, J. W. *Mem. geol. Soc. Am.* **67**, 773–782 (1957).
7. Porter, J. W. *Ecology* **53**, 745–748 (1972).
8. Barnes, D. J., *Bull. mar. Sci.* **23**, 280–289 (1973).
9. Barnes, D. J. & Taylor, D. L. *Helgol. Wiss. Meeresunters* **24**, 284–291 (1975).
10. Chalker, B. E. & Taylor, D. L. *Proc. R. Soc. B* **190**, 323–331 (1975).
11. Vosburgh, F. *Proc. 3rd int. Coral Reef Symp.*, **1**, 477–482 (1977).
12. Graus, R. R. *Proc. 3rd int. Coral Reef Symp.*, **2**, 464–469 (1977).
13. Jackson, J. B. C., in Larwood, G. & Rosen, B. R. (eds) *Biology and Systematics of Colonial Organisms*; *Syst. Assoc. Spec. Vol.* **11**, 499–555 (1979).
14. Porter, J. W. in *Growth, Maintenance, and Change of Coral Reefs* (ed. Barnes, D. J.) (in the press).
15. Pichon, M. *Proc. 2nd int. Coral Reef Symp.*, **2**, 55–68 (1974).
16. Adey, W. H. & Burke, R. *Bull. geol. Soc. Am.* **87**, 95–109 (1976).
17. Rosen, B. R. *Underwater Ass. Rep.* **1** (NS) 1–16 (1975).
18. Porter, J. W. *Am. Nat.* **110**, 731–742 (1976).
19. Connell, J. *Science* **199**, 1302–1310 (1978).
20. Komar, P. D. *Beach Processes and Sedimentation*, 1–429 (Prentice-Hall, Englewood Cliffs, 1976).
21. Chappell, J. *Bull. geol. Soc. Am.* **85**, 553–570 (1974).
22. Purdy, E. in *Reefs in Time and Space*, 9–76 SEPM Spec. Publ. (1974).
23. Shinn, E. A. *et al. Proc. 3rd int. Coral Reef Symp.*, **2**, 1–8 (1977).
24. Chappell, J. & Veeh, H. H. *Bull. geol. Soc. Am.* **89**, 356–368 (1978).
25. Chappell, J. & Thom, B. G. *Nature* **272**, 809–810 (1978).
26. Chappell, J. & Polach, H. *Bull. geol. Soc. Am.* **87**, 235–240 (1976).

A water depth model for the evolution of the planktonic Foraminiferida

Malcolm B. Hart

Department of Environmental Sciences, Plymouth Polytechnic, Drake Circus, Plymouth PL4 8AA, UK

Recent papers on the evolution of the planktonic Foraminiferida have stressed the importance of repeated patterns^{1,2} in the Neogene and Palaeogene, as well as in the Cretaceous^{1–4}. Most authors have recognized the importance of gross morphological characters (the biocharacters of Steineck and Fleischer²) and used them to illustrate the evolution of the superfamily Globigerinacea, as well as a prime tool in classification. Cifelli¹ and Frerichs⁴ relate such patterns of evolution to the available palaeotemperature data⁵, and while temperature is clearly important I shall show that it is not the complete story. Here the evolution of the planktonic Foraminiferida is reconsidered in the light of recent advances in the understanding of distributional controls of the modern fauna. I suggest that well-known 'iterative' trends indicate successive attempts at colonizing deeper levels in the water column.

In the planktonic environment, and especially in the surface waters where the planktonic Foraminiferida are most abundant, faunas avoid competition by adjusting their regional distributions to the different surface water and temperature regimes and by sometimes seeking different levels in the water column in their adult growth form. Bé⁶ has summarized his extensive knowledge of the modern fauna, and confirmed a threefold subdivision of the water column; shallow-water fauna (0–50 m), intermediate-water fauna (generally 50–100 m) and

deeper-water fauna (with adults normally below 100 m). This distribution can be identified in the Cretaceous using oxygen isotope data⁷ or, more conventional, stratigraphic information⁸.

Using planktonic:benthonic ratios (counted on the 500–250 µm grain-size-fraction) a depth curve⁸ has been produced for the Albian–Santonian interval of the Cretaceous succession in south-east England. A sample interval of 1 m (or less) was used throughout that study and the reliability of the graphs produced was tested by regional correlation^{9,10}. That water depth curve has many major features in common with other postulated depth curves for the same area^{11,12}, although there are some minor differences¹³. Onto this depth curve Hart and Bailey⁸ have reconstructed the evolutionary trends of the mid-Cretaceous planktonic Foraminiferida, placed at their predicted water depth (following the Bé⁶ model) and confirmed by their distribution in the shelf seas prevailing over north-west Europe at that time. In all the examples cited, mid-Cretaceous evolution either proceeds horizontally within the same depth zone, or migrates downwards into a lower depth zone. I know of no examples of evolution following a trend up into a higher level in the water column. As indicated by Hart and Bailey⁸, the distribution of an individual species and its appearance in time is, therefore, a function of the water depth available over the shelf during any particular interval. Differences in the known stratigraphic ranges of certain species are to be expected when one compares the UK succession with those recorded in the oceans by the Deep Sea Drilling Project¹⁴, or even areas like the Tethyan/Alpine region of Southern Europe¹⁵. Using this experience of the mid-Cretaceous fauna the central part of Fig. 1 was constructed, and then extended to include both the late Cretaceous/Cenozoic and the Triassic/Jurassic/early Cretaceous, with the aid of data from the literature^{1–6}. The depth preferences for each of the biocharacters is given where this is possible (following refs 6, 8), although this is clearly a great approximation the further one goes back through the geological record. There are also some characters that do not appear in all parts of the record, the most obvious being the lack of twin-keeled forms in the Palaeogene and Neogene, and the absence of biserial morphotypes at present.

Throughout the preparation of Fig. 1 the regional distributions of genera and species, together with their palaeoecological associations, have been considered. While the proposed model seems to cover most stratigraphic situations, there are clearly many cases where local palaeo-oceanographic conditions have created apparently anomalous occurrences of deeper-water morphotypes in what are regarded as shallow-water sediments (for example, *Globotruncana* spp. in the Campanian/Maastrichtian marginal calcarenites of Scania, S. Sweden; *Rotalipora* spp. in the Lower Middle Cenomanian calcareous sands of south-east Devon⁹; the apparently contradictory distribution of certain spinose/smooth species in parts of the Palaeogene; and the occurrence of some species of *Acarinina* and *Morozovella* in Palaeogene shallow-water sediments). Interesting, and important, as these individual cases may be, they do not negate the longer-term, overall trends outlined here.

Following my experience of mid-Cretaceous planktonic foraminiferal evolution⁸, it is possible to consider the complete Triassic to Recent time interval. Figure 1 shows the successive appearance of certain biocharacters exhibited by the whole superfamily Globigerinacea from its benthonic source (fully discussed by Masters¹⁶) in the Triassic(?). This original, primitive, shallow-water 'globigerinid' form remains virtually unchanged until the mid-Cretaceous (Aptian) when the first evolutionary explosion began. During, and following, the Aptian and Albian there was a very rapid appearance of new morphotypes of planktonic Foraminiferida, and their suggested depth stratification from this time onwards up the geological succession can be enclosed by the dotted line shown in Fig. 1, and contours the repeated conquest of deeper, surface, water habitats. This 'envelope' is also represented in Fig. 2, on which are also indicated the oceanic anoxic events of Schlanger and

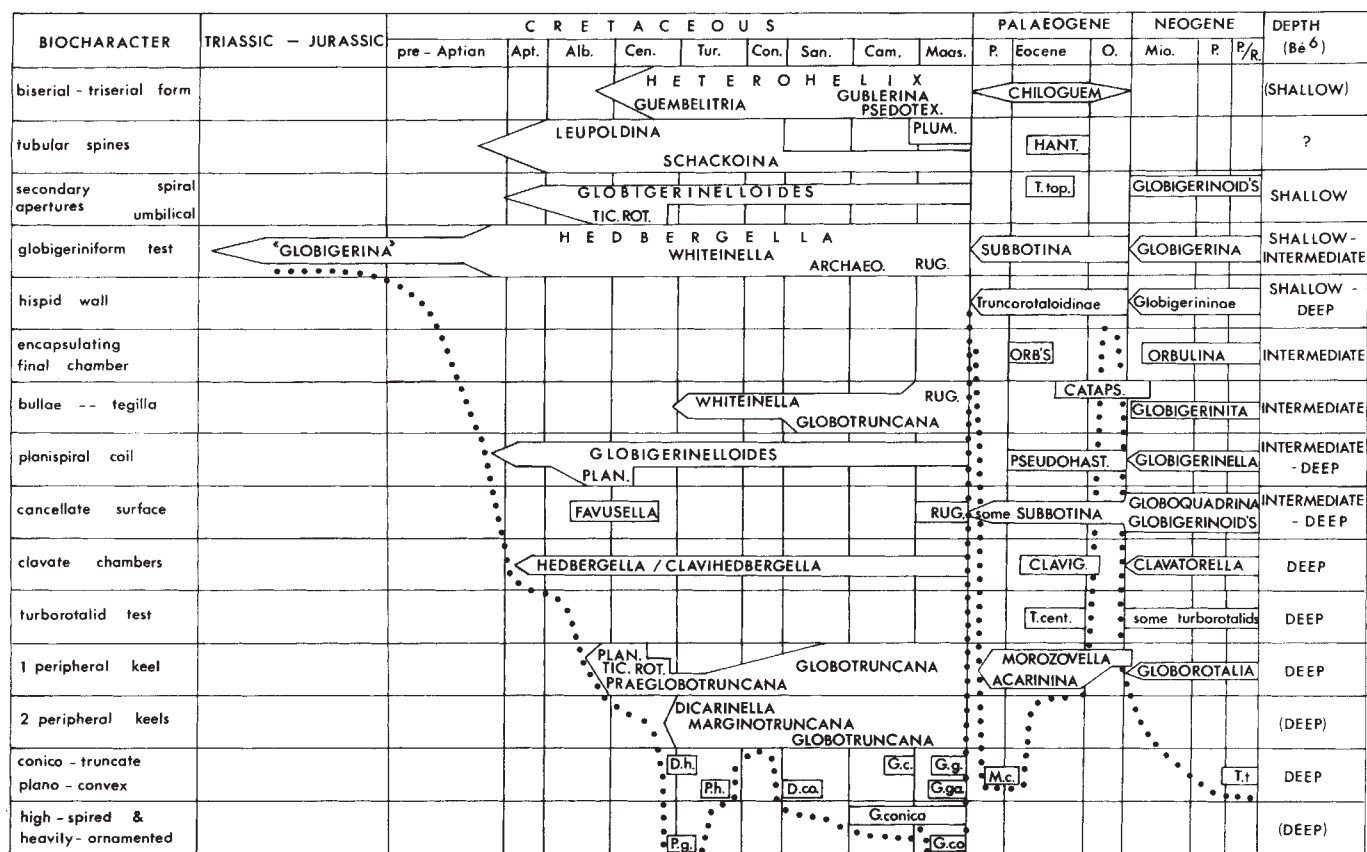
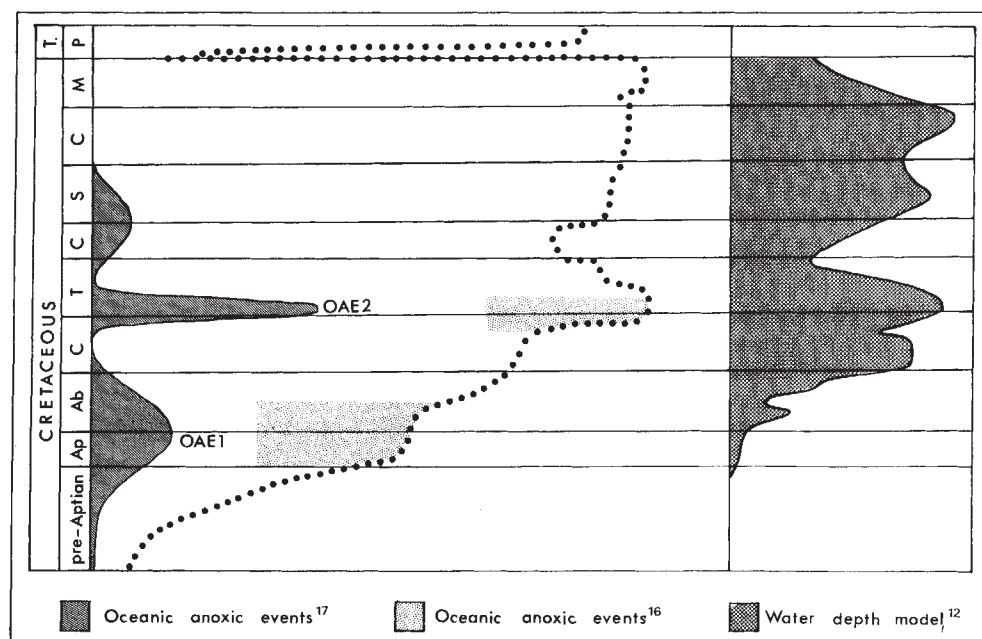


Fig. 1 Depth stratification model for the evolution of the planktonic Foraminiferida. *Pseudotex.*, *Pseudotextularia*; *Chiloguem.*, *Chiloguembelina*; *Plum.*, *Plummerita*; *Hant.*, *Hantkenina*; *Tic.*, *Ticinella*; *Rot.*, *Rotalipora*; *T. top.*, *Truncorotaloides topilensis* (Cushman); *Globigerinoid's*, *Globigerinoides*; *Archaeo.*, *Archaeoglobigerina*; *Rug.*, *Rugoglobigerina*; *Orb's*, *Orbulinoides*; *Cataps.*, *Catapsydrax*; *Plan.*, *Planomalina*; *Pseudohast.*, *Pseudohastigerina*; *Clavig.*, *Clavigerinella*; *T. cent.*, *Turborotalia centralis* (Cushman & Bermudez); *D.h.*, *Dicarinella hagni* (Scheibnerova); *P.h.*, *Praeglobotruncana helvetica* (Bolli); *G.c.*, *Globotruncana calcarata* Cushman; *G.g.*, *G. gagebini* Tiley; *G.ga.*, *G. gansseri* Bolli; *D.co.*, *Dicarinella concavata* (Brotzen); *M.c.*, *Morozovella caucasica* (Glaessner); *T.t.*, *Truncorotalia truncatulinoides* (d'Orbigny); *P.g.*, *Praeglobotruncana gibba* Klaus; and *G.co.*, *Globotruncana contusa* (Cushman).

Fig. 2 Depth stratification model compared with the transgression/regression model of Hancock¹² and Cretaceous anoxic events^{17,18}.



Jenkyns¹⁷ (together with the modifications proposed by Arthur and Schlanger¹⁸ and Jenkyns¹⁹). These anoxic events coincide with the levels of maximum faunal change (for example, the late Cenomanian–early Turonian boundary, the Aptian–Albian interval, and the Coniacian–Santonian interval). The ‘envelope’ is also similar, in broad outline, to many published transgression/regression models^{11,12}, especially that figured by Schlanger and Jenkyns¹⁷; the present curve is produced simply by matching foraminiferal test morphology against a Recent water depth scale⁶.

This close similarity suggests that the evolution of the planktonic Foraminiferida may be a function of increasing water depth on or near the shelf, which is, after all, where productivity is at its highest. This would be particularly true if a rising sea level was actively eroding its shorelines, thereby providing a vital nutrient source (Fig. 4 of ref. 19). This argument cannot, however, be applied to the Miocene–Pliocene interval where the same evolutionary patterns have been produced by a falling sea level. The same problem exists if one applies a temperature model; rising temperatures in the mid–late Cretaceous, and generally falling temperatures in the Miocene–Pliocene. Perhaps one should consider the stability of the stratification of the water column as the control. The oceanic anoxic events^{17–19} imply a certain degree of stagnation of the water column, probably created by overproduction of phytoplankton in the surface waters, and a lack of O₂-rich waters being introduced into the ocean basins from cold polar sources. In such a situation the oceans probably become very sluggish and highly stratified. Such a stratification of the water column immediately implies a host of physical/chemical constraints²⁰ including temperature. If the plankton in the water column were stimulated into high productivity by a rising sea level over the extensive Cretaceous shelf seas, there is the possibility that the associated O₂-depletion of the water could have contributed to the localized (and in some cases quite widespread) stagnation of the ocean floor (and the expanded shelf areas at times of high sea level) in a highly stratified situation.

During three main intervals of time (mid–late Cretaceous, Palaeogene and Neogene) the planktonic Foraminiferida apparently adapted (or were forced to adapt) to the status quo, and when the stratification was disturbed (by the terminal Cretaceous Event²¹, and the late Oligocene Event) they were then obliged to repeat their evolution as a direct response to the new situation.

This response has provided the successive appearance of the morphological features reported in Fig. 1 and the concomitant palaeotemperature values recorded by Douglas and Savin⁷. These various attempts to occupy the same levels in the water column have produced some remarkable examples of homeomorphy. Perhaps the best is the repeated evolution of the deeper-water plano-convex forms, with the successive appearance of the completely unrelated *Praeglobotruncana helvetica* (Bolli), *Dicarinella concavata* (Brotzen), *Globotruncana gansseri* Bolli, *G. gagnebini* Tilev, *Morozovella caucasica* (Glaessner), and *Truncorotalia truncatulinoides* (d'Orbigny). A not so extreme form of this morphotype (*Globotruncana stuartiformis* Dalbiez) was assigned to deeper levels in the water column on the basis of isotopic determinations by Douglas and Savin⁷.

While the proposed model (Fig. 1) is clearly for further discussion and research it does highlight many problems of foraminiferal classification. Can Masters¹⁶ be supported in his attempts to rejuvenate the use of *Globigerina* for Cretaceous species, when some workers in the Cenozoic feel unjustified in using that name back beyond the Neogene? It would also seem to indicate that in many places in the Cretaceous we are not using a phylogenetic classification, although the desirability/undesirability of such an approach is not being promoted by this account.

The recent work on isotopes/palaeotemperatures⁷ has suggested a depth stratification model for planktonic Foraminiferida as far back as the Albian, and this is supported here. Also,

if one could establish a direct relationship between eustatic changes (and their associated transgressions and regressions), phytoplankton productivity, evolution, and the production of anoxic sediments at selected levels in the geological record (particularly in the Cretaceous) then this would provide an interesting avenue of palaeo-oceanographic research.

The author acknowledges financial assistance from the School of Environmental Sciences Staff Research Fund, and the award of a research grant by the NERC. I also thank all my colleagues who have helped in the formulation of the above ideas.

Received 3 March; accepted 15 May 1980.

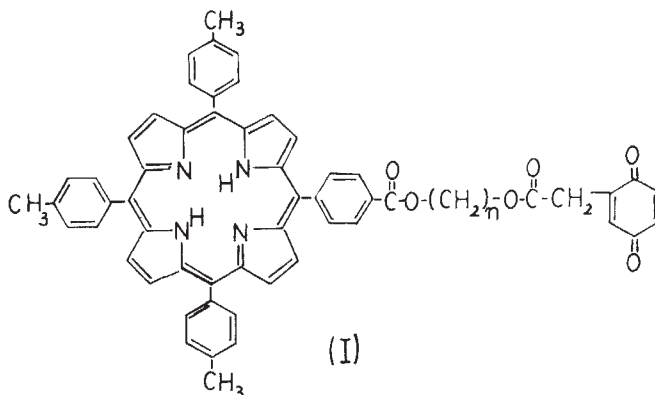
1. Cifelli, R. S. *Syst. Zool.* **18**, 154–168 (1969).
2. Steineck, P. L. & Fleischer, R. L. *J. Paleont.* **52**, 618–635 (1978).
3. McGowan, B. *Micropaleontology* **14**, 61–80 (1968).
4. Frerichs, W. E. *J. Paleont.* **45**, 963–968 (1971).
5. Emiliani, C. *Science* **154**, 851–857 (1966).
6. Bé, A. W. H. in *Oceanic Micropaleontology* Vol. 1 (ed. Ramsay, A. T. S.) 1–100 (Academic, London, 1977).
7. Douglas, R. G. & Savin, S. M. *Mar. Micropaleont.* **3**, 175–196 (1978).
8. Hart, M. B. & Bailey, H. W. *Aspekte der Kreide Europas* (ed. Weidmann, J.) 527–542 (IUGS, Ser. A, No. 6, 1979).
9. Carter, D. J. & Hart, M. B. *Bull. Br. Mus. nat. Hist. (Geol.)* **29**, 1–135 (1977).
10. Hart, M. B. & Carter, D. J. *J. Foramin. Res.* **5**, 114–126 (1975).
11. Cooper, M. R. *Palaeogeogr. Palaeoclimat. Palaeoecol.* **22**, 1–60, (1977).
12. Hancock, J. M. *Proc. geol. Ass.* **86**, 499–536 (1976).
13. Vail, P. R. *et al. Mem. Am. Ass. petrol. Geol.* No. 26 (1977).
14. Hart, M. B. *Cretaceous Research* Vol. 1 (in the press).
15. Robaszynski, F. & Caron, M. *Cahiers de Micropaleontologie* Part 1, 1–185; Part 2, 1–181 (1979).
16. Masters, B. A. *Oceanic Micropaleontology* Vol. 1 (ed. Ramsey, A. T. S.) 301–731 (Academic, London, 1977).
17. Schlanger, S. O. & Jenkyns, H. C. *Geol. Mijnb.* **55**, 179–184 (1976).
18. Arthur, M. A. & Schlanger, S. O. *Bull. Am. Ass. petrol. Geol.* **63**, 870–885 (1979).
19. Jenkyns, H. C. *J. geol. Soc.* **137**, 171–188 (1980).
20. Degens, E. T. & Stoffers, P. *Nature* **263**, 22–27 (1976).
21. Thierstein, H. R. & Berger, W. H. *Nature* **276**, 461–466 (1978).

Intramolecular photochemical electron transfer in a linked porphyrin–quinone molecule as a model for the primary step of photosynthesis

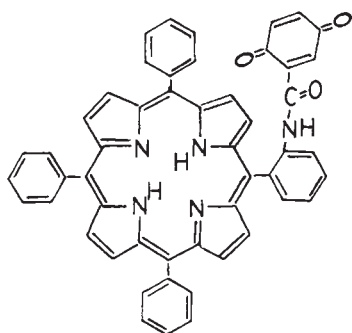
Te-Fu Ho, Alan R. McIntosh & James R. Bolton

Photochemistry Unit, Department of Chemistry, University of Western Ontario, London, Ontario, Canada N6A 5B7

The primary photochemical process in algal and green-plant photosynthesis is a one-electron transfer reaction from a chlorophyll donor species to an electron-acceptor molecule, with the electron transfer taking place across the thylakoid membrane probably within a special reaction-centre protein¹. A similar process takes place in bacterial photosynthesis. We have mimicked this reaction through the synthesis described here using the method of Kong and Loach², of a single molecule (P–Q) (I) containing a porphyrin (P) linked to a quinone (Q) by a hydrocarbon chain. When illuminated with visible light an intramolecular electron transfer occurs from the porphyrin to the quinone creating the biradical P^{•–}–Q^{•–} which can be detected by electron paramagnetic resonance (EPR) spectroscopy. In certain conditions the electron transfer is reversible.



There has been considerable interest in constructing various models to mimic the *in vivo* photochemical system. These have ranged from models of the primary donor and intermediate electron-acceptor complexes³⁻⁵, monolayer assemblies⁷⁻¹⁰, vesicles^{11,12}, intramolecular exciplex complexes¹³⁻¹⁸ to chlorophyll-quinone electron transfer in solution¹⁹. The P-Q compounds with $n = 2$ and 3 were previously synthesized by Kong and Loach². They reported that the benzoquinone oxidation state quenches the fluorescence of the attached porphyrin in dioxane and petroleum ether with a very low fluorescence yield in the latter solvent. Tabushi *et al.*²⁰ reported that in (II) fluorescence was considerably quenched compared to that of the unlinked porphyrin. They have attributed the fluorescence quenching to electron transfer but presented no direct evidence for such a mechanism.



(II)

In the synthesis of P-Q (I with $n = 3$) we followed the method of Kong and Loach² except that we started with the more stable 2,5-dimethoxyphenylacetic acid. The purity of the final compound was assessed by NMR and mass spectra which were consistent with (I) and by obtaining a single spot on thin layer chromatography using two separate solvents on silica gel. We also found that the fluorescence of P-Q ($n = 3$) in methanol at room temperature is quenched to an extent of 90% compared with that of the free porphyrin; however, this observation alone does not prove that photochemical electron transfer has taken place. More direct evidence can be obtained from EPR measurements. Both steady-state and transient EPR measurements were carried out for concentrations of P-Q between 10^{-4} and 10^{-3} M in a frozen methanol plus 2% chloroform solution between 12 and 165K. The P-Q UV-visible optical spectrum obeys Beer's law for concentrations up to 5×10^{-4} M. Above that there are some changes in the spectrum which could be due to dimerization. However, all measurements were carried out down to 10^{-4} M with little or no change in behaviour. For $T < 150$ K, photolysis with a 200 mW 647 nm Spectra Physics Krypton cw laser beam or with a Phase-R 620 nm pulsed dye laser generated an irreversible light-induced EPR signal. The spectrum (Fig. 1a) was found to be nearly invariant for temperatures below ~ 150 K. The g factor and the lineshape are similar to those of the unlinked porphyrin P^+ (Fig. 1c). However, on subtracting the P^+ spectrum from that of Fig. 1a (to the extent of one-half of the latter's integrated intensity), the difference spectrum of Fig. 1e is obtained, which is neither that of P^+ (Fig. 1c) nor the unlinked quinone Q^- (Fig. 1d). We have performed several control experiments in which the unlinked components P and Q were photolysed in methanol at low temperatures. When the concentration of Q is 1,000 times that of P, we observe irreversible intermolecular electron transfer, as shown in the EPR spectrum of Fig. 1b. When the spectrum of P^+ is subtracted from Fig. 1b in the same manner as before, the difference spectrum of Fig. 1f is generated. This spectrum is similar to that of the unlinked Q^- (see Fig. 1d) allowing for some spin exchange broadening effects. However, when P and Q are mixed at the same low concentration, the quantum efficiency of production of the EPR photolysis spectrum is much less than that generated by photolysis of the same

concentration (2×10^{-3} M) of the P-Q complex as shown in Fig. 2. Both meso-tetratolylporphyrin in chloroform/methanol and meso-tetra(4-carboxyphenyl)porphyrin in methanol were used, and nearly the same low yield of radicals was obtained.

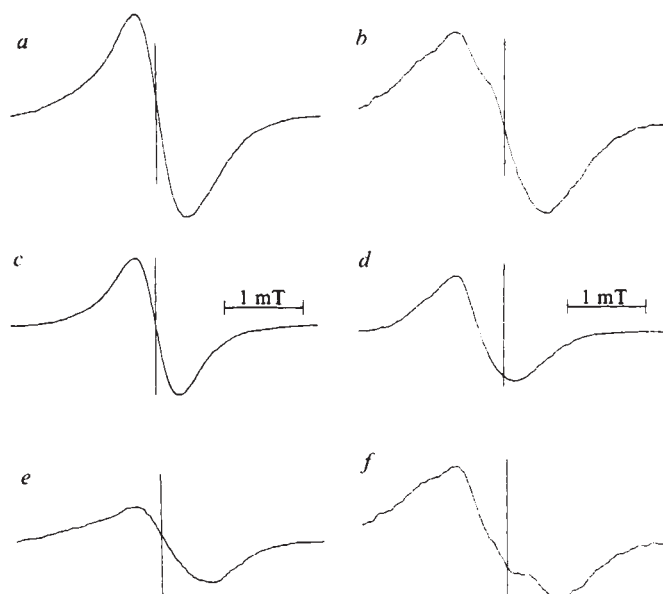


Fig. 1 Typical irreversible EPR spectra observed after the 200 mW 647 nm Krypton cw laser photolysis (except for c and d) of linked and non-linked P and Q compounds in methanol observed at 100K with EPR detection conditions of 0.2 mT modulation amplitude at 100 kHz and 1 mW microwave power at ~ 9.0 GHz. The vertical line in each case indicates the P^+ g factor: a, photolysis spectrum observed from the linked compound P-Q at 2×10^{-3} M concentration; b, photolysis spectrum observed from 10^{-4} M meso-tetra(4-carboxyphenyl)porphyrin with 10^{-1} M benzoquinone; c, meso-tetratolylporphyrin radical cation (obtained by iodine oxidation) at half of the integrated amplitude or concentration of part a; d, spectrum of semireduced quinone (obtained by the air oxidation of 2,5-dihydroxyphenylacetic acid) at half of the integrated amplitude or concentration of part a; e, difference spectrum of a (P-Q) minus c (P^+) which removes the theoretical 50% contribution of P^+ from the PQ photolysis spectrum; f, difference spectrum of b (P^+ and Q^-) minus c (P^+) which removes the theoretical 50% contribution of P^+ from the separated component spectrum.

On the basis of these steady-state EPR measurements, we conclude that the irradiation of P-Q results in an initial intramolecular electron transfer with high yield; however, there is also probably a low-yield contribution to the spectrum resulting from some electron transfer into the solvent or intermolecular transfer to the quinone moiety of another P-Q molecule.

As a further test of our intramolecular electron transfer hypothesis, we carried out flash-photolysis experiments with EPR detection²¹ as the temperature was raised from 90 to ~ 160 K (about 15° below the melting point of methanol). A large irreversible EPR signal was generated by photolysis at about 140K, as shown in Fig. 2. However, as the temperature approached 160K, the EPR kinetics became almost totally reversible, as shown in Fig. 3. The reversibility at 160K was very strong as the recorder pen of the Varian E-12 100 kHz EPR spectrometer jumped with each ~ 1 mJ flash of the red-filtered Photochemical Research Associates Model 610A Pulsed Light Source. The magnetic field profile of the EPR transients matched very well to the spectrum shown in Fig. 1a. Note that no reversible EPR signal could be observed by photolysis of solutions of the mixed unlinked components P and Q in conditions identical to those in Fig. 3 at 160K.

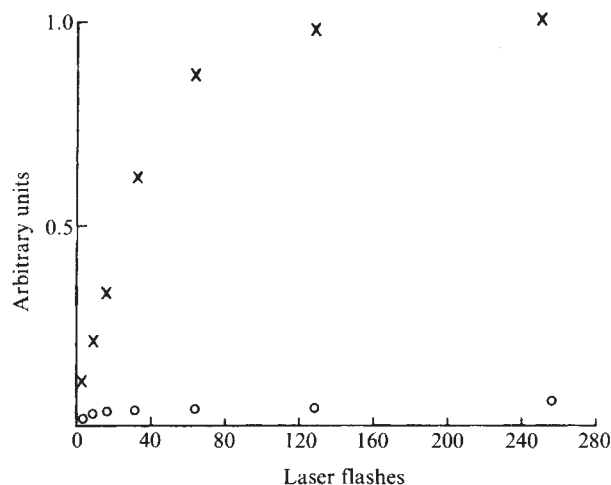


Fig. 2 Amplitude of the $g = 2.0$ EPR photolysis spectra induced by 620 nm photolysis at 140K in methanol followed by rapid cooling to 100K (where the spectra were measured as in Fig. 1) plotted as a function of the number of pulsed dye laser flashes with ~ 20 mJ per flash and a flashing rate of 1 Hz: for 2×10^{-3} M P-Q ($\times$); and for meso-tetra(4-carboxyphenyl)porphyrin and benzoquinone each at 2×10^{-3} M ($\circ$).

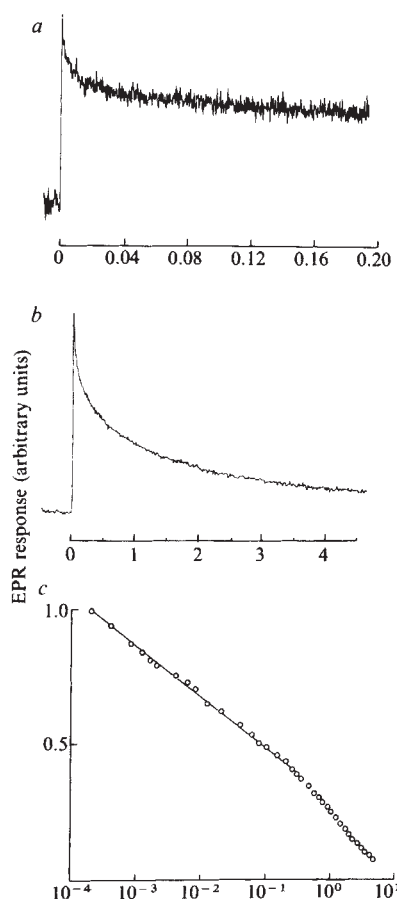


Fig. 3 Typical flash induced reversible EPR kinetics observed at 160K at the first derivative low field maximum of the spectrum of Fig. 1a for 2×10^{-3} M P-Q in methanol. The pulsed xenon light source was filtered by a Corning CS-262 filter with ~ 1 mJ per flash and the flashing rate was 0.1 Hz with 256 flashes accumulated in each kinetic trace and with EPR detection conditions of 0.5 mT modulation amplitude at 100 kHz and 5 mW microwave power at ~ 9.0 GHz: a, time domain of 200 ms; b, time domain of 5 s; c, logarithmic time domain up to 5 s.

Note that the observed electron-transfer kinetics (see Fig. 3) are approximately logarithmic in time over nearly four orders of magnitude, as shown in Fig. 3c. Ke *et al.*²² have carried out a quantitative analysis of the charge recombination in photosystem I of green plants at low temperatures, and have shown that $\log t$ kinetics can be observed for an intramolecular electron-transfer process. They showed that the $\log t$ kinetics can be explained by assuming a rectangular distribution function for the distances separating the reacting species, or for some other parameter, such as the activation energy, for which the rate constant is an exponential function. On the basis of the restricted temperature domain for the observations of electron-transfer reversibility in the present work, we assume that we are observing a rectangular distribution in activation energies rather than a distribution of donor-acceptor distances leading to a temperature-independent electron tunnelling expression. Thus, at $\sim 15^\circ$ below the melting point of methanol, we assume that there is a range of activation energies for the electron to return to the primary donor arising from the relative motion of the donor and acceptor ends of P-Q at this temperature. This range of activation energies could arise from the various conformations of the hydrocarbon chain linking the quinone acceptor to the porphyrin donor.

We have used the route of Kong and Loach² to synthesize a donor-acceptor-molecule which, in certain conditions, exhibits reversible photochemical intramolecular electron transfer. Similar photochemical reactivity for P-Q has been observed at room temperature in acetonitrile (J.L.Y. Kong and P. A. Loach, personal communication). This system is an excellent model for the primary photochemical electron-transfer process in reaction centres of photosynthesis as both the confinement of donor and acceptor together and reversible photochemical electron transfer have been duplicated. This well-characterized model system should give us considerable insight into the mechanism of the *in vivo* reaction. We are continuing these studies with a view to stabilize the charge separation reaction and to study the effect of distance and molecular structure on the kinetics and mechanism of the intramolecular electron-transfer process.

This research was supported by a Strategic Grant in Energy from the Natural Sciences and Engineering Research Council of Canada. We thank Professor P. A. Loach for helpful discussions. This is publication no. 242 of the Photochemistry Unit.

Note added in proof: It has come to our attention that Dalton and Milgrom²³ have prepared porphyrins with attached quinones. However, they only report substantial fluorescence quenching.

Received 14 February; accepted 9 May 1980.

1. Sauer, K. in *Bioenergetics of Photosynthesis* Chap. 3, 115-180 (ed. Govindjee) (Academic, New York, 1975).
2. Kong, J. L. Y. & Loach, P. A. in *Frontiers of Biological Energetics—Electrons to Tissues*, Vol. 1 (eds Dutton, P. L., Leigh, J. S. & Scarpa, A.) 73-82 (Academic, New York, 1978). Abstract No. THPMA12 of the 7th Annual Meeting of the American Society for Photochemistry, Asilomar (1979); *J. Het. Chem.* (in the press).
3. Katz, J. J. in *Light Induced Charge Separation in Biology and Chemistry* (eds Gerischer, H. & Katz, J. J.) 331-359 (Dahlekonferenzen, Verlag Chemie, Berlin, 1979).
4. Wasielewski, M. R. in *Frontiers of Biological Energetics—Electrons to Tissues* Vol. 1 (eds Dutton, P. L., Leigh, J. S. & Scarpa, A.) 63-72 (Academic, New York, 1978).
5. Pellin, M. J., Kaufmann, K. J. & Wasielewski, M. R. *Nature* **278**, 54-55 (1979).
6. Boxer, S. G. & Bucks, R. R. *J. Am. chem. Soc.* **101**, 1883-1885 (1979).
7. Kelly, J. M. & Porter, G. *Proc. R. Soc. A* **319**, 319-329 (1979).
8. Kuhn, H. in *Light-Induced Charge Separation in Biology and Chemistry* (eds Gerischer, H. & Katz, J. J.) (Dahlekonferenzen, Verlag Chemie, Berlin 1979).
9. Janzen, A. F., Bolton, J. R. & Stillman, M. J. *J. Am. chem. Soc.* **101**, 6337-6341 (1979).
10. Janzen, A. F. & Bolton, J. R. *J. Am. chem. Soc.* **101**, 6342-6348 (1979).
11. Ford, W. E., Otvos, J. W. & Calvin, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3590-3593 (1979).
12. Kurihara, K., Sukigara, M. & Toyoshima, Y. *Biochim. biophys. Acta* **547**, 117-126 (1979).
13. Chandross, E. A. & Thomas, H. T. *Chem. phys. Lett.* **9**, 393-396 (1971).
14. Okada, T. *et al. Chem. phys. Lett.* **14**, 563-568 (1972).
15. Ide, R., Sakata, Y. & Misumi, S. *JCS Chem. Commun.* 1009 (1972).
16. Gnädig, K. & Eiselthal, K. B. *Chem. phys. Lett.* **46**, 339-342 (1977).
17. McIntosh, A. R., Manikowski, H. & Bolton, J. R. *J. phys. Chem.* **83**, 199-219 (1978).
18. Migita, M., Kawai, M., Mataga, N., Sakata, Y. & Misumi, S. *Chem. phys. Lett.* **53**, 67-70 (1978).
19. Tollin, G. *Bioenergetics* **6**, 69-87 (1974); *J. phys. Chem.* **80**, 2274-2277 (1976).
20. Tabushi, I., Koga, N. & Yanagita, M. *Tetrahedron. Lett.* No. 3, 257-260 (1979).
21. McIntosh, A. R., Manikowski, H. & Bolton, J. R. *J. phys. Chem.* **83**, 3309-3314 (1979).
22. Ke, B., Demeter, S., Zamarev, K. I. & Khairutdinov, R. F. *Biochim. biophys. Acta* **545**, 264-284 (1979).
23. Dalton, J. & Milgrom, L. R. *JCS Chem. Commun.* 609 (1979).

Inhibition of photosynthesis in *Arabidopsis* mutants lacking leaf glutamate synthase activity

C. R. Somerville & W. L. Ogren*

Department of Agronomy, University of Illinois, Urbana, Illinois 61801

The CO_2 released in photorespiration is believed to result from a complex mitochondrial reaction in which glycine is converted to equimolar amounts of CO_2 , NH_3 and the C-1 group of $\text{N}^5, \text{N}^{10}$ -methylene-tetrahydrofolate¹⁻³. Because photorespiratory CO_2 production may amount to $80 \mu\text{mol per h per g fresh weight}$ ^{4,5} the stoichiometry of the decarboxylation reaction indicates that NH_3 release from glycine exceeds primary NO_3^- reduction^{6,7}. Leaf cells must therefore be capable of rapid NH_3 re-assimilation in photorespiratory conditions. It has been suggested⁸ that NH_3 could be refixed directly into glutamate by mitochondrial glutamate dehydrogenase (GDH), utilizing the NADH generated during glycine decarboxylation^{9,10}. This seems unlikely^{11,12} because of the high K_m for NH_3 exhibited by GDH *in vitro*. An alternative suggestion^{11,12} that photorespiratory NH_3 could be re-assimilated into glutamate by the sequential action of cytoplasmic glutamine synthetase (GS) and the chloroplast enzyme glutamate synthase (GOGAT)^{13,14} is supported by circumstantial evidence from *in vitro* studies^{12,15}. Here we provide direct evidence for such a pathway, based on the results of experiments with mutants of *Arabidopsis thaliana* (L.) Heynh which are deficient in leaf GOGAT activity.

These mutants are inviable in atmospheric conditions that permit photorespiration, but are fully viable and phenotypically indistinguishable from the wild type when maintained in an atmosphere that suppresses photorespiration⁵ (1% CO_2 in air). This conditional lethal phenotype, shared by several other mutants with defects in photorespiratory carbon metabolism^{16,17}, provided the basis for the isolation of the mutants¹⁶.

Leaf extracts of wild-type *Arabidopsis* contained levels of ferredoxin-dependent GOGAT activity (Table 1) comparable to that observed in other species^{11,18}. NADH or NADPH (0.2 mM) were relatively ineffective as electron donors, supporting about 4% of the activity obtained with ferredoxin in otherwise identical assay conditions (results not presented). Leaf extracts of the mutant lines CS30, CS103 and CS113 contained less than 5% of the wild-type level of ferredoxin-dependent GOGAT activity (Table 1). Mixing of mutant and wild-type extracts resulted in additive activity, suggesting that the low activity of mutant extracts was not due to the presence of an inhibitor. The presence of normal levels of ribulose biphosphate (RuBP) carboxylase activity in all extracts indicates that recovery of chloroplast enzymes was similar in each case. Extracts of the mutants also contained normal levels of activity for GDH, GS and several enzymes related to photorespiration (results not presented). Thus, the mutants seem to be specifically defective in the synthesis of functional leaf GOGAT.

A single nuclear mutation seems to be responsible for the enzyme deficiency and the conditional lethal phenotype. Reciprocal F_1 hybrids from crosses of wild-type and mutant lines showed the wild-type phenotype and had about 50% of wild-type GOGAT activity (Table 1). The mutant phenotype, scored as the onset of chlorosis after 4 days exposure to standard atmospheric conditions^{15,16}, segregated 3:1 (wild type:mutant) in the F_2 progeny resulting from crosses of wild-

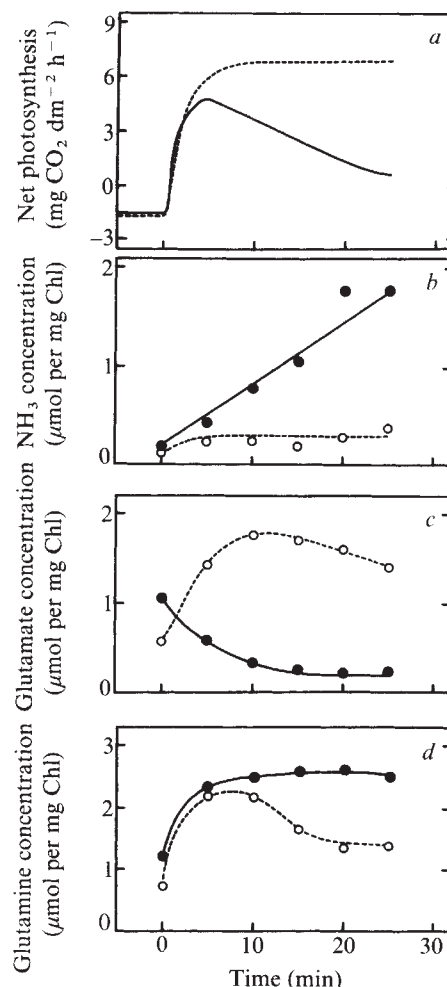


Fig. 1 Changes in *a*, photosynthesis rate and levels of *b*, NH_3 ; *c*, glutamate; and *d*, glutamine during illumination of wild-type and mutant *Arabidopsis* in photorespiratory conditions. Plants were grown in a 1% CO_2 atmosphere¹⁶ on an artificial medium irrigated with a solution containing 2.5 mM KH_2PO_4 (pH 6.5), 5 mM KNO_3 , 2 mM $\text{Ca}(\text{NO}_3)_2$, 2 mM MgSO_4 and micronutrients. The plants were placed in darkness in standard atmospheric conditions several hours before use to allow equilibration of metabolite pools. The plants were then placed in a photosynthesis chamber at 25 °C and continuously flushed with a gas stream ($357 \mu\text{l l}^{-1} \text{CO}_2$, 50% O_2 , balance N_2) which exited through an infrared gas analyser. At time zero, the plants were illuminated at $300 \mu\text{Einsteins m}^{-2} \text{s}^{-1}$. At the indicated intervals, plants were removed from the chamber and the leaf material ground in 2 ml chloroform/methanol/water (5:12:1). An additional 1.5 ml of chloroform and 1 ml of water was added, and the phases separated by centrifugation. Chlorophyll was determined on an aliquot of the organic phase. NH_3 was assayed in an aliquot of the aqueous phase by a colorimetric procedure²¹. Amino acid concentrations were determined with a Beckman (Model-119CL) amino acid analyser. Mutant and wild-type averaged 1.8 mg chlorophyll per g fresh weight. The response of the *gluS* mutant strain CS113 is indicated by the solid lines and symbols (●) and that of the wild type by broken lines and open symbols (○). Chl, chlorophyll.

type × mutant. F_1 hybrids, obtained by crossing the mutants in all possible combinations, showed the mutant phenotype and were deficient in GOGAT activity (see for example CS103 × CS113, Table 1). The lack of complementation is evidence that the mutants carry defective alleles of the same gene, which we have designated *gluS*. Co-segregation of the mutant phenotype and enzyme deficiency was tested by first scoring the F_2 progeny from a wild-type × CS113 cross for the mutant phenotype at the rosette stage. The plants were revived by transfer to a CO_2 -enriched environment for 2 weeks, then 64

* Also at US Department of Agriculture, Science and Education Administration-Agricultural Research, Urbana, Illinois 61801.

randomly chosen plants were assayed individually for GOGAT. We observed an absolute correspondence between low enzyme activity and mutant phenotype.

The photosynthetic characteristics of the *gluS* mutants are similar to those of *Arabidopsis* mutants deficient in phosphoglycolate phosphatase¹⁶ and serine-glyoxylate aminotransferase¹⁷. In atmospheric conditions that limit the oxygenation of RuBP⁵ (1% CO₂ and 21% O₂ or 350 μ l l⁻¹ CO₂ and 2% O₂), photosynthesis (carbon dioxide fixation) continued indefinitely at wild-type rates (results not presented). However, in photorespiratory conditions (357 μ l l⁻¹ CO₂, 50% O₂, balance N₂), the photosynthesis rate of the mutants was initially similar to the wild type but rapidly declined to about 7% of that rate (Fig. 1a). The inhibition was reversible, for if the mutants were subsequently shifted to atmospheric conditions which suppressed photorespiration, photosynthesis was fully restored within about 30 min. Photosynthesis was also restored, irrespective of atmospheric conditions, by placing the plants in darkness for a similar period.

The inhibition of photosynthesis and reduced viability of the mutants in standard atmospheric conditions may be attributed to the accumulation of high levels of NH₃ during illumination in photorespiratory conditions (Fig. 2b). During 25 min of illumination in 357 μ l l⁻¹ CO₂, 50% O₂, balance N₂, free NH₃ in mutant CS113 increased 20-fold to a final intracellular concentration of about 3 mM. In the same conditions the concentration of NH₃ in the wild type remained below about 0.4 mM. The high concentration of NH₃ in the mutants would be expected to cause the observed inhibition of photosynthetic CO₂ fixation by uncoupling photophosphorylation¹⁹. In conditions of high CO₂ or low O₂, carbon flow through the photorespiratory pathway is negligible⁵ so that little NH₃ is released from glycine. Photosynthesis and growth of the *gluS* mutants are therefore unimpaired in conditions which prevent photorespiration.

The accumulation of NH₃ in the *gluS* mutant in photorespiratory conditions is attributed to the virtual

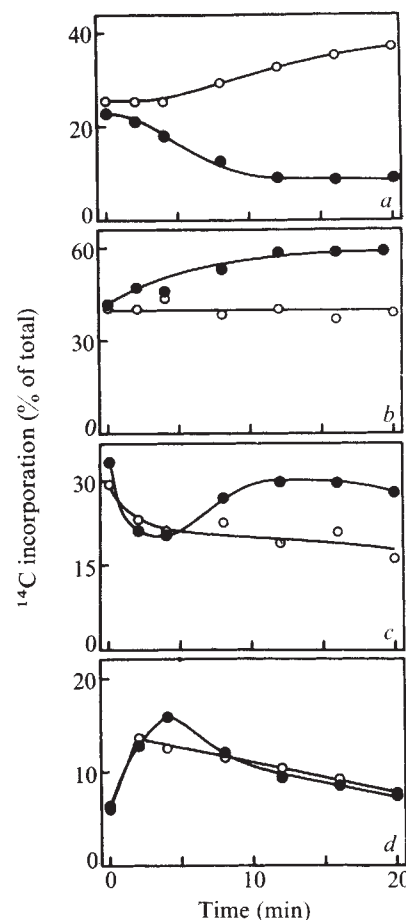


Fig. 2 Products of ¹⁴CO₂ assimilation by wild-type and mutant *Arabidopsis*. Intact plants were placed in a photosynthesis chamber and flushed with a humidified gas stream containing 350 μ l l⁻¹ CO₂, 21% O₂, balance N₂. At time zero the plants were illuminated (300 μ Einsteins m⁻² s⁻¹). At the indicated intervals the system was closed and ¹⁴CO₂ introduced. After 2 min the plants were quickly (3–5 s) removed to liquid nitrogen. The labelled products were extracted and separated as described previously¹⁶. The CO₂ concentration in the chamber remained between 300 and 350 μ l l⁻¹ during the period of incorporation. a, Basic fraction (amino acids); b, acid-1 fraction (organic acids and sugar monophosphates); c, combined acid-2 and acid-3 fractions (mostly sugar diphosphates); d, neutral fraction (sugars). The response of the mutant strain CS113 is indicated by solid symbols and that of the wild type by open symbols.

Table 1 Glutamate synthase activity in extracts of wild-type and mutant lines of *Arabidopsis*

Strain	Glutamate synthase activity*	RuBP carboxylase activity*
Wild type	5.3	30.4
CS30	0.2	29.7
CS103	0.2	29.7
CS113	0.1	30.7
F ₁ (W1 × CS113)	2.8	31.1
F ₁ (CS103 × CS113)	0.3	31.5
Mixture†	2.8	30.0

Extracts were prepared by grinding leaf material in 50 mM KH₂PO₄ (pH 7.2), 12.5 mM β -mercaptoethanol, 0.5 mM EDTA and 0.5 mM EGTA. The extracts were centrifuged (30,000 g for 15 min), passed through a column of Sephadex G-75, and dialysed against the same buffer for 12 h. Glutamate synthase activity was determined by measuring the formation of ¹⁴C-glutamate from labelled 2-oxoglutarate. The complete 500 μ l assay mixture¹⁸ contained: 25 μ mol KH₂PO₄ (pH 7.2), 6.2 μ mol β -mercaptoethanol, 0.25 μ mol EDTA, 0.25 μ mol EGTA, 5 μ mol aminooxyacetate, 1 μ mol L-glutamine, 1 μ mol ¹⁴C-oxoglutarate (0.5 μ Ci μ mol⁻¹), 50 μ g spinach ferredoxin (Sigma), 0.8 mg Na₂S₂O₄, 0.8 mg NaHCO₃ and 60–70 μ g of extract protein. The reaction was initiated by the addition of the dithionite and bicarbonate and terminated after 10 min at 24 °C by the addition of 60 μ l of 3% H₂O₂. Product formation was measured by liquid scintillation counting following fractionation of the labelled compounds by ion exchange chromatography. Control reactions, lacking reductant, produced 1% of the d.p.m. obtained in the complete reaction mixture. RuBP carboxylase activity was measured as described previously¹⁶. Protein was determined by a dye binding assay²⁰.

*Enzyme activity is expressed as μ moles of product per h per mg protein.

†Equal volumes of wild-type and mutant (CS113) extract were mixed immediately before assay.

disappearance of free glutamate in leaf tissue (Fig. 1c), and thus the lack of an amino acceptor for re-assimilation of NH₃ by GS. The reduction in glutamate corresponds roughly to the increase in glutamine (Fig. 1d) as NH₃ is initially re-fixed by GS. The glutamine pool in the mutant then stabilizes because further synthesis is prevented by the lack of glutamate, and the GOGAT deficiency prevents conversion of glutamine to glutamate. The observation that NH₃ is not rapidly re-fixed into glutamate verifies the suggested¹¹ low *in vivo* activity of GDH in an assimilatory direction and confirms the importance of the GS/GOGAT pathway in the re-assimilation of photorespiratory NH₃^{11,12}.

The low intracellular concentration of glutamate caused a plethora of secondary metabolic aberrations evident from the pattern of ¹⁴CO₂ incorporation into the products of photosynthesis (Fig. 2). In this experiment, plants were allowed to photosynthesize in standard atmospheric conditions for various periods, then given a short (2 min) pulse of ¹⁴CO₂ and the photosynthetic products were analysed. During the first few minutes, the labelling pattern of mutant and wild type were essentially indistinguishable. However, after several

minutes of photosynthesis, the proportion of label in the amino acid fraction of the mutant dropped considerably (Fig. 2a). This is attributed to reduced availability of glutamate as an amino donor for the synthesis of glycine from glyoxylate. As a result, the intracellular concentration of serine declined to about one-fifth of the wild-type level (data not presented) as serine was utilized, by way of serine-glyoxylate aminotransferase, for glycine synthesis.

The central role of glutamate in cellular metabolism precludes a succinct description of the manifold effects resulting from glutamate depletion. In this respect it is not surprising that the mutants are inviable in normal atmospheric conditions. More surprising, perhaps, is the normal growth of the mutants in non-photorespiratory conditions. This implies that the plant does not require leaf GOGAT activity for NH_3 assimilation from primary nitrate reduction. Rather, the sole requisite function of leaf GOGAT seems to be the re-assimilation of photorespiratory NH_3 .

We thank R. H. Hageman for growth chamber facilities, J. M. Widholm for amino acid analyses, and M. E. Hageman for technical assistance. This research was supported in part by grant No. 5901-0410-9-0341-0 from the USDA Competitive Research Grants Office.

Received 10 March; accepted 16 April 1980.

- Bird, I. F., Cornelius, M. J., Keys, A. J. & Whittingham, C. P. *Phytochemistry* **11**, 1587-1594 (1972).
- Kisaki, T., Yoshida, N. & Imai, N. *Pl. Cell Physiol.* **12**, 275-288 (1971).
- Canvin, D. T., Lloyd, N. D. H., Fock, H. & Przybylla, K. in *CO₂ Metabolism and Plant Productivity* (eds Burris, R. H. & Black, C. C.) 161-176 (University Park Press, Baltimore, 1976).
- Keys, A. J., Sampaio, E. V. S. B., Cornelius, M. J. & Bird, I. F. *J. exp. Bot.* **28**, 525-533 (1977).
- Chollet, R. & Ogren, W. L. *Bot. Rev.* **41**, 137-179 (1975).
- Brunetti, N. & Hageman, R. H. *Pl. Physiol.* **58**, 583-587 (1976).
- Millin, B. J. *Planta* **105**, 225-233 (1972).
- Tolbert, N. E. *Encyclopedia Pl. Physiol.* (New Ser.) **6**, 338-352 (1979).
- Douce, R., Moore, A. L. & Neuburger, M. *Pl. Physiol.* **60**, 625-628 (1977).
- Woo, K. C. & Osmond, C. B. *Aust. J. Pl. Physiol.* **3**, 771-785 (1976).
- Lea, P. J. & Millin, B. J. *Encyclopedia Pl. Physiol.* (New Ser.) **6**, 445-456 (1979).
- Keys, A. J. *et al. Nature* **275**, 741-743 (1978).
- Lea, P. J. & Millin, B. J. *Nature* **251**, 614-616 (1974).
- Wallsgrave, R. M., Lea, P. J. & Millin, B. J. *Pl. Physiol.* **63**, 232-236 (1979).
- Anderson, J. W. & Done, J. *Pl. Physiol.* **60**, 354-359 (1977).
- Somerville, C. R. & Ogren, W. L. *Nature* **280**, 833-836 (1979).
- Somerville, C. R. & Ogren, W. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2684-2687 (1980).
- Wallsgrave, R. M., Harel, E., Lea, P. J. & Millin, B. J. *J. exp. Bot.* **28**, 588-596 (1977).
- Good, N. *Biochim. biophys. Acta* **40**, 502-517 (1960).
- Spector, T. *Analyt. Biochem.* **86**, 142-146 (1978).
- McCullough, H. *Clin. chim. Acta* **17**, 297-304 (1967).

Regulation of wound ethylene synthesis in plants

Thomas Boller* & Hans Kende†‡

*Botanisches Institut der Universität Basel, 4056 Basel, Switzerland

†Institut für Allgemeine Botanik, Eidgenössische Technische Hochschule, 8092 Zürich, Switzerland

Ethylene is produced by plants at specific stages of their life cycle and is involved in the regulation of many developmental processes, such as fruit ripening, flower fading, leaf abscission, growth in aquatic plants and initiation of flowering in bromeliads^{1,2}. Ethylene is also formed when plants are subjected to stress by, for example, wounding, noxious chemicals, drought or waterlogging¹⁻³. In both cases the biosynthetic pathway begins with methionine¹⁻⁵. It proceeds to ethylene by way of S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC), a recently discovered intermediate^{6,7}, in ripening fruit^{7,8} and auxin-treated pea stem sections^{9,10}. We now report that wound-induced ethylene synthesis also proceeds by way of ACC and that it is regulated at the level of the ACC-forming enzyme (ACC synthase). Wounding of pericarp tissue of tomato fruit (*Lycopersicon esculentum* Mill.) results in rapid enhancement of the activity of ACC synthase and, consequently, in a greatly increased rate of ACC synthesis.

Cut tomato fruit evolves large amounts of wound ethylene¹¹⁻¹³. We found that disks excised from the pericarp of green tomatoes produced relatively little ethylene in response to isolation (Table 1), but when each disk was further cut into 12 sectors (wounding), there was a substantial increase in ethylene production. In the same tissue, production of ACC increased even more rapidly as a result of wounding (Table 1). Aminoethoxyvinylglycine (AVG) is a potent inhibitor of ethylene biosynthesis² and of ACC synthase from tomato fruit⁸. When sectors of pericarp were incubated on 0.1 mM AVG after wounding, ethylene production was inhibited 89% after the first hour (2.07 nmol per g per h in control compared with 0.23 nmol per g per h in AVG-treated sectors).

In further experiments, we determined the activity of ACC synthase at short intervals after wounding of pericarp tissue and correlated the results with the time course of ACC accumulation and ethylene production. The activity of ACC synthase, as determined *in vitro*, was low in freshly cut sectors, tripled within 20 min and increased 10-fold within 40 min (Fig. 1), remaining fairly constant thereafter. The amount of ACC in the tissue increased approximately linearly after a lag of about 20 min; thus, the accumulation rate of ACC *in vivo* essentially paralleled the activity of ACC synthase *in vitro* (Fig. 1). The activity of ACC synthase was only about 20% of that expected from the rate of *in vivo* ACC synthesis, and we have been unable to discover where the loss occurred. Finally, the rate of ethylene formation, calculated from the total formed after wounding, increased after a lag of 30 min for at least 100 min and was proportional to the amount of ACC in the tissue.

Table 1 Effect of wounding on ethylene and ACC production

Incubation time (h)	Treatment	Ethylene		ACC	
		nmol per g	nmol per g per h	nmol per g	nmol per g per h
0		—	—	1.27	—
1	Intact	0.12	0.12	3.81	2.54
1	Wounded	0.72	0.72	8.73	7.46
2	Intact	0.25	0.13	3.77	—
2	Wounded	3.04	2.32	18.40	9.67

Disks of 11.5 mm diameter were cut from the pericarp of glasshouse-grown green tomato fruit (cv. Linda) and trimmed to 3 mm by removing extra tissue that formerly faced the inside of the fruit. They were incubated in pairs, epidermis facing upwards, on moist filter paper in 25-ml serum vials sealed with rubber stoppers at 28 °C. Disks were wounded by cutting each into 12 equal sectors. Ethylene was determined by gas chromatography¹⁵. ACC was extracted with ice-cold ethanol (4 ml for two disks), and insoluble residue was removed by centrifugation. The supernatant was taken to dryness and the residue was redissolved in 1 ml water. Of this, 0.1 ml was tested in the ACC assay of Lizada and Yang¹⁶, based on the liberation of ethylene from ACC in alkaline solution containing Hg^{2+} and NaOCl . We had verified that all material in the crude ethanolic extract which released ethylene in the ACC assay of Lizada and Yang co-chromatographed with authentic ACC on cellulose thin-layer plates (solvent 2 of Boller *et al.*⁸).

The tissue already had the capacity to liberate ethylene from ACC during the lag phase, as reflected by the high rate of ethylene production in freshly cut sectors incubated on 10 mM ACC (Fig. 2). The rate of ethylene production during the first 10 min after cutting was 0.66 nmol per g per h and increased to a steady rate of about 2 nmol per g per h within 80 min. The maximum rate of ethylene formation was reached faster in tissue on 10 mM ACC than in that on 1 mM ACC. Attainment of the maximum rate of conversion of ACC to ethylene was probably delayed because of the time required to take up ACC into the pericarp sectors. The maximum rate of ACC-dependent ethylene formation might have been limited by

‡Permanent address: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824.

saturation of an ACC uptake system and/or by saturation of the ethylene-forming system.

Ethylene synthesis after wounding, as in other situations, is inhibited by cycloheximide^{5,14}. When sectors of pericarp were incubated on cycloheximide ($25 \mu\text{g ml}^{-1}$) for 2 h, the tissue produced 86% less ethylene and contained 84% less ACC and 96% less ACC synthase activity than untreated sectors.

These results indicate that ethylene is produced from ACC in tomato pericarp tissue in response to wounding. Such ethylene formation seems to be induced through rapid enhancement of ACC synthase activity. Because cycloheximide inhibits the development of this enzyme activity, it is likely,

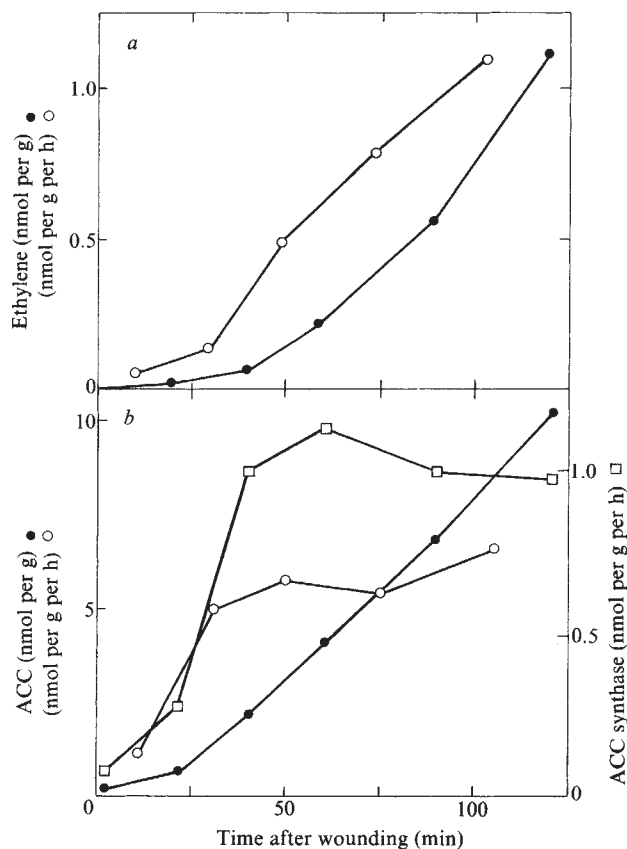


Fig. 1 Ethylene evolution, ACC content and ACC synthase activity in wounded pericarp disks from green tomato fruit. *a*, Ethylene production. From each of two freshly harvested tomato fruits (cv. Moneymaker), seven disks were cut. Each was wounded immediately by cutting into 12 equal sectors. Pairs of wounded disks, one from each fruit, were incubated for different times on moist filter paper in sealed 25-ml serum vials. After incubation, ethylene was determined^{1,5}, and the tissue was frozen in liquid N_2 . Data are mean values from three identically incubated samples. The rate of ethylene production was calculated for each time interval of the experiment. *b*, ACC content and activity of ACC synthase. The frozen sectors from *a* (six disks, 1.8 g fresh weight total) were combined, pulverized in liquid N_2 with pestle and mortar and extracted in 3 ml 0.1 M K-HEPES buffer, pH 8, containing 4 mM dithiothreitol (DTT) and $0.4 \mu\text{M}$ pyridoxal phosphate. After centrifugation (10 min at $10,000g$), two 0.1-ml samples of supernatant solution were used directly for ACC determination¹⁶. The rate of increase in ACC content was derived for each time interval of the experiment. The true rates of ACC formation were probably 10% higher if all ethylene formed was derived from ACC. Centrifuged extract (2 ml) was desalted on a column of Sephadex G-50 (2×6 cm) with 2 mM K-HEPES buffer, pH 8, containing 0.1 mM DTT and $0.2 \mu\text{M}$ pyridoxal phosphate as eluting buffer, and the eluted protein was used to determine ACC synthase activity with $50 \mu\text{M}$ SAM as substrate⁸. Enzymatically formed ACC was determined according to Lizada and Yang¹⁶.

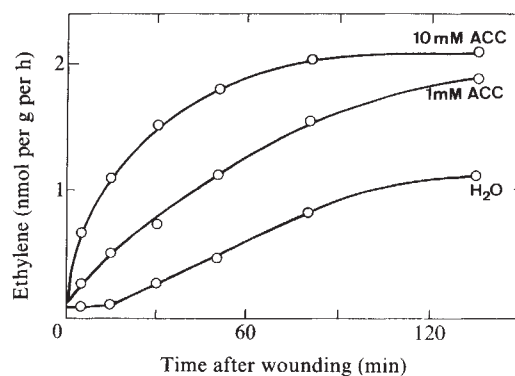


Fig. 2 Rate of ethylene formation in wounded pericarp incubated on ACC. Freshly cut disks of pericarp from a single tomato (cv. Linda) were cut into 12 sectors each, and the sectors from two disks were incubated in sealed 25-ml serum vials on filter paper wetted with water or 1 mM or 10 mM solutions of ACC. Ethylene was determined by gas chromatography¹⁵ after 10, 20, 40, 60, 100 and 170 min. From these data, the mean rate of ethylene formation was calculated for each time interval. Control vials containing ACC solutions without tissue produced less than $2 \mu\text{mol h}^{-1}$ ethylene.

though not proven, that ACC synthase is newly formed rather than activated when the tissue is wounded. The last step in ethylene formation—conversion of ACC to ethylene—does not seem to be regulated in wounded tissue. This follows from two kinds of experiments. First, wounded pericarp disks already had the capacity to convert added ACC to ethylene during the lag phase of wound-induced ethylene synthesis. Second, when no ACC was added, the rate of ethylene formation was proportional to the level of endogenous ACC throughout the experiment. This also indicates that the system converting ACC to ethylene is not saturated at the concentrations of ACC reached in the wounded tissue.

The regulation of wound ethylene synthesis in tomato pericarp resembles that of ethylene synthesis in senescing tomatoes. In the latter case, the activity of ACC synthase and the concentration of ACC increase during maturation of the fruit, when ethylene formation is initiated⁸. Similarly, auxin at high concentrations seems to induce ethylene synthesis in etiolated pea epicotyls by enhancing ACC synthase activity⁹. It remains to be seen whether developmental, stress and hormonal stimuli all regulate the activity of ACC synthase through the same mechanism or whether different modes of control are exerted to modulate the activity of this important enzyme in ethylene synthesis.

We thank Professor Ph. Matile, in whose laboratory this work was carried out, for his hospitality, Dr G. Défago for plant material and Mr P. Mötteli for technical assistance. The work was supported in part by NSF grant PCM 77-08522 to H.K.

Received 11 February; accepted 2 May 1980.

1. Abeles, F. B. *Ethylene in Plant Biology* (Academic, New York, 1973).
2. Lieberman, M. A. *Rev. Pl. Physiol.* **30**, 533–591 (1979).
3. Yang, S. F. & Pratt, H. K. in *Biochemistry of Wounded Plant Tissues* (ed. Kahl, G.) 595–622 (Walter de Gruyter, Berlin, 1978).
4. Hanson, A. D. & Kende, H. *Pl. Physiol.* **57**, 538–541 (1976).
5. Hyodo, H. *Pl. Physiol.* **59**, 111–113 (1977).
6. Lürssen, K., Naumann, K. & Schröder, R. Z. *Pflanzenphysiol.* **92**, 285–294 (1979).
7. Adams, D. O. & Yang, S. F. *Proc. natn. Acad. Sci. U.S.A.* **76**, 170–174 (1979).
8. Bolter, T., Herner, R. C. & Kende, H. *Planta* **145**, 293–303 (1979).
9. Jones, J. F. & Kende, H. *Planta* **146**, 649–656 (1979).
10. Konze, J. R. & Kende, H. *Planta* **146**, 293–301 (1979).
11. Meigh, D. F., Norris, K. H., Craft, C. C. & Lieberman, M. *Nature* **186**, 902–903 (1960).
12. Lee, T. H., McGlasson, W. B. & Edwards, R. A. *Radiat. Bot.* **10**, 521–529 (1970).
13. Herner, R. C. & Sink, K. C. Jr *Pl. Physiol.* **52**, 38–42 (1973).
14. Saltveit, M. E. Jr & Dilley, D. R. *Pl. Physiol.* **64**, 417–420 (1979).
15. Kende, H. & Hanson, A. D. *Pl. Physiol.* **57**, 523–527 (1976).
16. Lizada, C. & Yang, S. F. *Analyt. Biochem.* **100**, 140–145 (1979).

Heterozygosity at enzyme loci and morphological variation

Paul Handford*

Rockefeller University, New York City 10021

*Present address: Zoology Department, University of Western Ontario, London, Canada N6A 5B7

Two recent reports^{1,2} have concerned the relationship between genic heterozygosity, as indicated by electrophoretically detected enzyme loci, and an individual's tendency to depart from the mean on a variety of morphological traits. Using different methods to assess differences in morphological variances between homozygote and heterozygote classes, both conclude that homozygotes show a significantly higher level of morphological variation. These conclusions are important in that they seem to extend the phenomenon from studies on manipulated stocks of organisms³ to variation in natural populations. As Eanes² points out, it is necessary to determine the generality of this result. Both these studies^{1,2} consider poikilotherm animals (a fish and an insect), so it would be particularly interesting to look for the relationship in homoiotherms. As a contribution to this end, I present here the results (Table 1) of an analysis of variation in the Rufous-collared sparrow, *Zonotrichia capensis*⁴. I found no difference in levels of variation either between the homozygote and heterozygote classes or between classes of individuals heterozygous at different numbers of loci. My results thus suggest a difference between homoiotherms and poikilotherms in this phenomenon.

My data concern variation at four enzyme loci (mannose-6-phosphate dehydrogenase, M6PI; 6-phosphogluconate dehydrogenase, 6-PGD; glucose-6-phosphate dehydrogenase, G6PD and α -glycerophosphate dehydrogenase, α -GPD) and in 11 metrical characters (tarsus, tail length and three bill, four toe and two wing measures). As the sexes differ significantly on several variables they are considered separately.

There are some difficulties in comparing levels of variation among population samples or subsamples. These and some

possible solutions are reviewed by Van Valen⁵. In the univariate case, one customarily compares sample variances. Additional variables may justifiably be considered independently, provided there is low correlation between them. This is the approach adopted by Eanes², considering variation in two wing characters in the butterfly, *Danaus plexippus*. It is unwise, however, to test the null hypothesis, σ^2 homozygotes = σ^2 heterozygotes, by use of an F -test. The F -distribution is rather sensitive to deviations from normality of the measured distributions, which can give spuriously high values of F . The simplest way to test such a null hypothesis is that given by Levene⁶. One constructs a new variable $y_i = |x_i - \bar{x}|$ for each case i and tests the samples for differences in y by means of a t -test.

In most circumstances it is probably best to consider variation in several characters collectively, by some multivariate method, rather than separately, as even small degrees of correlation between variables introduce potential bias through, in effect, making the same test several times. This is particularly relevant when using the sign test⁷ to assess a hypothesis. Mitton¹ has used two techniques for extracting a single measure of variability from his seven meristic characters: computing the mean coefficient of variation, c.v., and the determinant of the variance-covariance matrices, $|\Sigma|$. These are compared among the homozygote and heterozygote classes, and the results tested by the sign test. In fact, matrices may be tested for homogeneity of dispersion, and a probability ascribed⁸, but one should note some undesirable properties of $|\Sigma|$ as a statistic of multivariate variability. If even one of the characters used in computing $|\Sigma|$ has a very low variance, then $|\Sigma|$ becomes very small regardless of variation in other characters. Small differences in such very small numbers will have large effects on the ratios between them, and so interpretation of these ratios becomes difficult and potentially misleading. Similarly, any degree of correlation between variables, which is almost inevitable when using more than three or four variables, reduces $|\Sigma|$ to very small values. Table 1 shows the results of applying a multivariate test for matrix homogeneity. Three of five tests among males are significant, but probably spurious given the very tiny values of $|\Sigma|$ (of the order of 10^{-20}). Van Valen⁵ also discusses the use of principal components in this kind of analysis. This approach eliminates the problem of correlated variables, but exaggerates the problem of low variance in one or more variables, as the last principal components to be extracted

Table 1 Comparison of variability in morphological characters among genetic subgroups in samples of Rufous-collared sparrows, *Zonotrichia capensis*

	Loci									
	M6PI		6PDG		G6PD		α -GPD		Heterozygosity	
	Hom	Het	Hom	Het	Hom	Het	Hom	Het	Low	High
Males										
N	76	49	103	22	74	38	88	37	68	44
Σs^2	0.054	0.070	0.059	0.061	0.062	0.063	0.060	0.060	0.055	0.075
Hom/Het	0.782 NS		0.962 NS		0.983 NS		1.000 NS		0.739 NS	
Matrix homogeneity, P	0.041*		0.372 NS		0.037*		0.321 NS		0.008†	
Females										
N	37	10	36	11	37	9	41	6	21	25
Σs^2	0.059	0.043	0.059	0.042	0.058	0.055	0.058	0.039	0.063	0.52
Hom/Het	1.387 NS		1.398 NS		1.059 NS		1.502 NS		1.196 NS	
Matrix homogeneity, P	—		0.000†		—		—		0.585 NS	

Heterozygosity is the number of loci, of four, at which an individual is heterozygous. In males, Low = 0–1 loci; in females, Low = 0 loci. Different criteria were used to make sample sizes comparable. Σs^2 is the simple sum of all character variances within given subgroups. Hom/Het is the ratio $\Sigma s^2_{\text{hom}}/\Sigma s^2_{\text{het}}$; significance level tested by Levene's method (see text). Matrix homogeneity is the P level given by a multivariate test for the homogeneity of the two variance-covariance matrices. Where no value is given, as in the females, this is because the test could not be carried out due to matrix singularity caused by there being more morphological variables than cases in the heterozygote class. NS, Not significant. All analyses performed on ln-transformed measurements.

usually account for a very small fraction of the total variance in the sample. There are no objective methods available appropriate to the present purpose, for setting a level for exclusion of these later components, and in any case this loses information.

By analogy with the univariate case, Van Valen recommends the use of total sample variance for j variables, $\Sigma_j s_j^2$. This eliminates problems of correlation and low variances by making them irrelevant. If the variables are log-transformed, scale effects are eliminated as is the need for some multivariate c.v. Differences in Σs^2 may again be tested for significance by an extension of Levene's test: $y_i = (\Sigma_j (x_{ij} - \bar{x}_j)^2)^{1/2}$, again using a t -test for differences in $\bar{y}$. The results of such an analysis of my data fail to show significantly different levels of variation either between the homozygote and heterozygote classes considered separately, or between classes of individuals heterozygous at different numbers of loci. Because the studies of Mitton¹ and Eanes² use other methods of analysis, it is not possible to make a close comparison between their results and mine. However, some points are worth mentioning. First, the absence of a significant result in this single sample from one species does not, of course, demonstrate the absence of the effect. What it does is raise the possibility of a difference between homoiotherms and poikilotherms in this regard, one which is consistent with other considerations about development and adaptation, as indicated below. It has been widely suggested that development of homoiotherms is more resilient to environmental insult than that of poikilotherms and invertebrates, and that their growth rates and final size are more determinate⁹. Again, genetic enzyme polymorphism among vertebrates as a whole is lower than among invertebrates, and homoiotherms are slightly, though non-significantly, less variable than are poikilotherm vertebrates¹⁰. This perhaps reflects different tactics of adaptation among very different kinds of organisms, and suggests the possibility that heterozygosity and polymorphism may have quite different significance in organisms with widely different organization and ecological situations¹¹. This specifically suggests a relationship between levels of genic heterozygosity, body size and mobility which is consistent with predictions from Levins' theory of adaptive strategies in relation to environmental grain¹². Thus, vertebrates which are typically larger and more mobile, so experiencing the environment in a more fine-grained fashion, exhibit lower levels of genic polymorphism. There are other studies considering variability and heterozygosity (at the population level), but these also consider variation in poikilotherms and invertebrates^{13,14}. Clearly, there is a need for investigation of variation in a wider range of taxa, particularly the higher vertebrates, before we can make generalizations about the relationship between genic heterozygosity and morphological variability.

This work was supported by NSF grant GB38344 to Fernando Nottebohm. I thank Roger Green, Shiva Singh and the reviewers for their comments.

Vitamin D₃ metabolites and PTH synergistically stimulate bone formation of chick embryonic femur *in vitro*

Hiroyoshi Endo*, Mamoru Kiyoki†, Kohtaro Kawashima*, Tatsuyuki Naruchi† & Yoshinobu Hashimoto†

*Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

†Teijin Institute for Bio-Medical Research, 4-3-2 Asahigaoka, Hino-shi, Tokyo 191, Japan

Bone remodelling depends on a balance between formation and resorption. Little is known of the biological factors involved in bone formation, whereas there is much evidence that physiological factors, such as parathyroid hormone (PTH) and active metabolites of vitamin D₃, influence resorption. Cells responsible for osteogenesis and osteoclasts occur in close proximity, suggesting that both might be controlled by the same physiological factors in the micro-environment of the body fluid. To clarify the factors stimulating bone formation or accelerating osteogenic cell activity, we have examined the effects of vitamin D₃ metabolites and PTH on bone formation *in vitro*. We report here that combinations of those metabolites especially 1 α ,25-(OH)₂D₃ and 24R,25-(OH)₂D₃, directly stimulate calcification on bone synergistically with PTH.

We used the culture system described before^{1,2}. Femora of 9-day-old chick embryos were obtained by removing adherent soft tissues under a dissecting microscope. Each femur was transferred to a roller tube containing 1.5 ml of medium. To allow for differences between embryos, the bones from one side of an embryo were cultivated in control medium and those from the opposite side of the same embryo in experimental medium. Basal medium was a mixture of BGJb-HW2 (ref. 3) and 11-day-old chick embryo extract (CEE), (9:1). PTH (1 U ml⁻¹), 1 α ,25-(OH)₂D₃ (0.02 and 2.0 ng ml⁻¹), 24R,25-(OH)₂D₃ (0.5 and 50.0 ng ml⁻¹) and 25-OH-D₃ (2.5 ng ml⁻¹) were added to the mixture, singly or in combination, to examine their effect on calcification. Roller tubes were incubated at 37 °C for 6 days, rotating 10 times per hour in a roller drum which held them at an angle of 5°. The medium was replaced every other day. At the end of cultivation, the calcium of most pair-mate bones was determined and

Table 1 Calcium level of 9-day-old chick embryonic femur after 6 days cultivation using PTH added to the basal medium as control

Treatment	Concentration (ng ml ⁻¹)	No. of pairs	Calcium level (μ g per femur)		
			Control bones	Treated bones	Calcification ratio*
1 α ,25-(OH) ₂ D ₃	0.02				
+ 24R,25-(OH) ₂ D ₃	0.5	11	4.42 $\pm$ 0.74	7.54 $\pm$ 0.79	2.07 $\pm$ 0.16†
+ 25-OH-D ₃	2.5				
1 α ,25-(OH) ₂ D ₃	0.02				
+ 24R,25-(OH) ₂ D ₃	0.5	13	4.40 $\pm$ 0.63	7.71 $\pm$ 0.60	2.04 $\pm$ 0.24†
1 α ,25-(OH) ₂ D ₃	0.02				
+ 25-OH-D ₃	2.5	10	5.61 $\pm$ 0.56	5.71 $\pm$ 0.57	1.05 $\pm$ 0.09
24R,25-(OH) ₂ D ₃	0.5				
+ 25-OH-D ₃	2.5	8	7.65 $\pm$ 0.69	8.93 $\pm$ 0.43	1.15 $\pm$ 0.11
1 α ,25-(OH) ₂ D ₃	0.02	6	5.03 $\pm$ 0.79	5.55 $\pm$ 0.99	1.05 $\pm$ 0.06
1 α ,25-(OH) ₂ D ₃	2.0	5	6.60 $\pm$ 1.12	6.96 $\pm$ 0.79	1.38 $\pm$ 0.25
24R,25-(OH) ₂ D ₃	0.5	5	10.88 $\pm$ 1.21	11.13 $\pm$ 1.12	1.06 $\pm$ 0.05
24R,25-(OH) ₂ D ₃	50.0	5	8.74 $\pm$ 1.21	8.14 $\pm$ 0.83	0.97 $\pm$ 0.08
25-OH-D ₃	2.5	5	5.65 $\pm$ 1.74	5.80 $\pm$ 1.46	1.00 $\pm$ 0.13

Values represent mean $\pm$ s.e.m. The amount of calcium deposited in the femur was determined by OCP method¹⁵. Control femur was cultivated in the basal medium plus PTH.

*The ratio of calcium levels in treated femora to pair-mate control bones.
† $P < 0.005$.

Received 20 November 1979; accepted 11 April 1980.

- Mitton, J. B. *Nature* **273**, 661-662 (1978).
- Eanes, W. F. *Nature* **276**, 263-264 (1978).
- Lerner, I. M. *Genetic Homeostasis* (Oliver and Boyd, Edinburgh, 1954).
- Handford, P. & Nottebohm, F. *Evolution* **30**, 802-817 (1976).
- Van Valen, L. *J. theor. Biol.* **45**, 235-247 (1974); *Evolutionary Theory* **4**, 33-43 (1978).
- Levene, H. in *Contributions to Probability and Statistics* (eds Olkin, I. et al.) 278-292 (Stanford University Press, 1960).
- Sokal, R. R. & Rohlf, F. J. *Biometry* (Freeman, San Francisco, 1969).
- Cohen, E. & Burns, P. *SPSS-MANOVA-Multivariate Analysis of Variance and Covariance* (Document No. 413, Rev. A, Northwestern University, Evanston, 1977).
- Case, T. J. *Q. Rev. Biol.* **35**, 243-282 (1978).
- Nevo, E. *Theor. Pop. Biol.* **13**, 121-177 (1978).
- Selander, R. K. & Kaufman, D. W. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1875-1977 (1973).
- Levins, R. *Evolution in Changing Environments* (Princeton University Press, 1968).
- Soulé, M. E. *Taxon* **20**, 37-50 (1971); *Am. Nat.* **106**, 429-446 (1972); *Evolution* **33**, 396-401 (1979).
- Soulé, M. E. & Baker, B. *Heredity* **23**, 611-614 (1968).

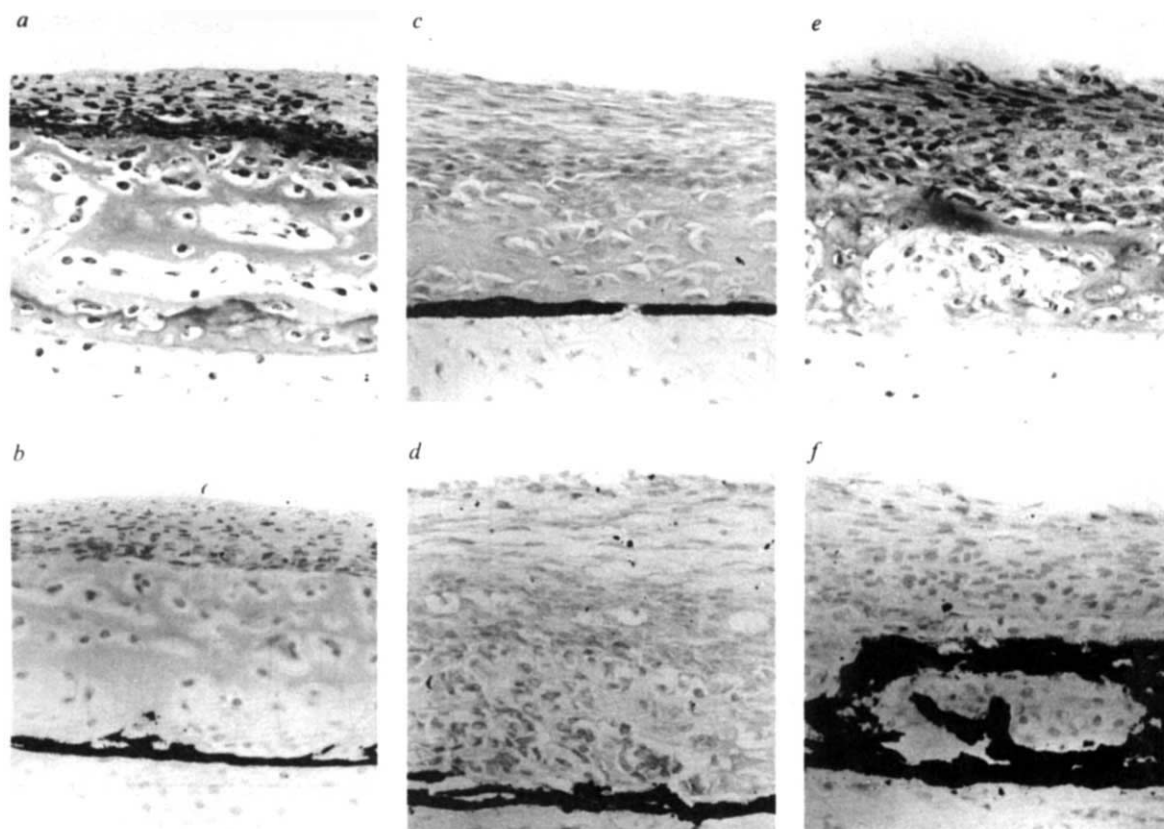


Fig. 1 Diaphyses of the femora from 9-day chick embryos after 6 days cultivation. *a*, Bone cultured in the medium CEE:BGJb-HW2 (1:9) (haematoxylin and eosin stain); *b*, the same specimen as in *a* (von Kossa's stain); *c*, bone cultured in medium containing the three metabolites of vitamin D₃ (von Kossa's stain); *d*, bone cultured in medium containing PTH (von Kossa's stain); *e*, bone cultured in medium containing PTH and the three metabolites of vitamin D₃ (haematoxylin and eosin stain); *f*, the same specimen as in *e* (von Kossa's stain). All $\times 400$.

histological development of residual pairs was examined microscopically with haematoxylin–eosin and von Kossa's stain.

Femora cultured in basal medium or in medium with vitamin D₃ metabolite(s) formed only osteoid tissue with no calcification (Fig. 1*a–c*). In medium containing PTH alone, mineralization scarcely occurred (Fig. 1*d*), although the bone elongated significantly⁴. In medium plus PTH and three kinds of vitamin D₃ metabolites, however, the diaphyseal cortex of the bone became heavily calcified (Fig. 1*e, f*).

The amount of calcium deposited in the femur treated with all three vitamin D₃ metabolites in the presence of PTH was 2.07 times greater than that in control bone treated with PTH alone. Similarly, combined treatment with 1 α ,25-(OH)₂D₃ and 24R,25-(OH)₂D₃ in the presence of PTH gave a value of 2.04 for that calcification ratio. When combined with 25-OH-D₃ in the presence of PTH, however, neither 1 α ,25-(OH)₂D₃ nor 24R,25-(OH)₂D₃ stimulated calcium deposition, the calcification ratio being 1.05 and 1.15 (Table 1). Moreover, no single treatment with 1 α ,25-(OH)₂D₃ or 24R,25-(OH)₂D₃ in the presence of PTH affected calcification, even at high concentrations (Table 1). 1 α ,25-(OH)₂D₃, when combined with 24R,25-(OH)₂D₃, could be replaced by the synthetic vitamin D₃ analogue 1 α ,24R-(OH)₂D₃ (ratio 1.93), although the synthetic vitamin alone with added PTH did not enhance calcification (ratio 1.03). Bone growth in medium plus PTH alone was 1.04 times that in medium alone, and in medium plus three kinds of vitamin D₃ metabolite the ratio was 1.17 (Table 2). Treatment with any one of these vitamin D₃ metabolites alone did not affect calcification.

Many observations made *in vivo* have suggested that vitamin D₃ metabolites could be involved in bone mineralization⁵. Our results show that these metabolites, especially the combination of 1 α ,25-(OH)₂D₃ and 24R,25-

(OH)₂D₃, stimulate bone formation under the synergistic influence of PTH. These can activate bone resorption^{6,7}, and so our result suggested that bone could be remodelled successfully by simultaneous activation of both osteoclastic and osteogenic processes.

1 α ,25-(OH)₂D₃ is the most active metabolite of vitamin D₃ for many biological processes⁸, and we have now demonstrated that 24R,25-(OH)₂D₃ is important for ossification, with mineralization proceeding only in the presence of both metabolites. This supports *in vivo* studies which suggested that 24R,25-(OH)₂D₃ may be active in bone

Table 2 Calcium level of 9-day-old chick embryonic femur after 6 days cultivation using basal medium as control

Treatment	Concentration (ng ml ⁻¹)†	No. of pairs	Calcium level (µg per femur)		
			Control bones	Treated bones	Calcification ratio*
PTH	1†	11	6.35 ± 1.00	6.89 ± 0.81	1.04 ± 0.13
1 α ,25-(OH) ₂ D ₃	0.02				
+ 24R,25-(OH) ₂ D ₃	0.5	7	9.60 ± 1.15	10.27 ± 0.58	1.17 ± 0.15
+ 25-OH-D ₃	2.5				
1 α ,25-(OH) ₂ D ₃	0.02				
+ 24R,25-(OH) ₂ D ₃	0.5	6	6.45 ± 1.47	7.31 ± 1.43	1.14 ± 0.06
1 α ,25-(OH) ₂ D ₃	0.02	5	10.06 ± 0.30	10.12 ± 0.85	1.01 ± 0.07
1 α ,25-(OH) ₂ D ₃	2.0	5	9.84 ± 1.46	8.84 ± 1.46	0.93 ± 0.11
24R,25-(OH) ₂ D ₃	0.5	5	10.40 ± 0.39	9.52 ± 0.76	0.91 ± 0.05
24R,25-(OH) ₂ D ₃	50.0	5	9.92 ± 0.87	8.76 ± 0.27	0.92 ± 0.10

Values represent mean $\pm$ s.e.m. The amount of calcium deposited in the femur was determined by OCP method¹⁵. Control femur was cultivated in the basal medium.

*The ratio of calcium level in treated femora to pair-mate control bone.

†PTH concentration was 1 unit ml⁻¹.

formation^{9,10}, possibly in combination with $1\alpha,25\text{-(OH)}_2\text{D}_3$ (ref. 11).

The role of PTH in bone formation, however, has been controversial, with some studies suggesting that it inhibits bone collagen synthesis¹², and other evidence indicating that it increases bone formation^{13,14}. Our results demonstrate that PTH is essential for the calcification of bone. We are now studying the role of CEE and the dose-response relationship of PTH and vitamin D_3 metabolites in stimulating bone formation *in vitro*.

Received 2 August 1979; accepted 16 April 1980.

1. Endo, H. *Expl. Cell Res.* **21**, 151-163 (1960).
2. Ito, Y., Endo, H., Enomoto, H., Wakabayashi, K. & Takamura, K. *Expl. Cell Res.* **31**, 119-127 (1963).
3. Endo, H. *Connective Tissue* **6**, 139-148 (1974).
4. Kawashima, K., Iwata, S. & Endo, H. *Bone Metabolism* **6**, 114-117 (1973).
5. De Luca, H. F. in *Vitamin D Metabolism and Function* (Springer, Berlin, 1979).
6. Margroub, A. & Sheppard, H. *Endocrinology* **100**, 629-634 (1977).
7. Robertson, W. F., Peacock, M., Atkins, D. & Webster, L. A. *Clin. Sci.* **43**, 715-718 (1972).
8. De Luca, H. F. & Schnoes, H. K. *A. Rev. Biochem.* **45**, 631-666 (1976).
9. Orony, A., Goodwin, D., Noff, D. & Edelstein, S. *Nature* **276**, 517-519 (1978).
10. Kanis, J. A. *et al. Br. med. J.* **1**, 1382-1386 (1978).
11. Molluche, H. H. *et al. in Vitamin D: Basic Research and its Clinical Application*, 425-427 (De Gruyter, Berlin, 1979).
12. Dirtrich, J. W., Canalis, E. M., Maina, D. M. & Raisz, L. G. *Endocrinology* **98**, 943-949 (1976).
13. Kalu, D. N., Pennock, J., Doyle, F. H. & Foster, G. V. *Lancet* **i**, 1363-1366 (1970).
14. Flanagan, B. & Nichols, G. J. *clin. Invest.* **44**, 1795-1801 (1964).
15. Connerty, H. V. & Briggs, A. R. *Am. J. clin. Path.* **45**, 290-296 (1966).

Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes

A. W. Ford-Hutchinson, M. A. Bray, M. V. Doig, M. E. Shipley & M. J. H. Smith

Biochemical Pharmacology Research Unit, Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London SE5 8RX, UK.

Arachidonic acid is metabolised either by cyclooxygenases to produce prostaglandins and thromboxanes or by lipoxygenases to produce mono-, di- and trihydroxyeicosatetraenoic acids (HETEs). Polymorphonuclear leukocytes (PMNs) release HETEs, including mono- and dihydroxy fatty acids, when exposed to stimuli such as the calcium ionophore A23187 (refs 1, 2). The mono-HETEs are assumed to be of particular importance with respect to effects on leukocyte function because they have been shown to possess both chemotactic and chemokinetic activities towards PMNs and eosinophils^{3,4}. However, we have now shown that the chemokinetic and aggregating activities released from rat and human PMNs exposed to ionophore A23187 (ref. 5) are not due to the release of mono-HETEs but to that of 5,12-di-HETE (leukotriene B). This compound is active over the concentration range 10 pg ml^{-1} to 5 ng ml^{-1} .

Rat peritoneal or human peripheral PMNs, when exposed to ionophore A23187, release substances into the surrounding media which cause the aggregation and chemokinesis of other PMN suspensions⁵. The release of the activities was prevented by drugs known to inhibit lipoxygenase pathways of arachidonic acid (AA) metabolism.

The aggregating and chemokinetic activities released following exposure of human PMNs to ionophore A23187 were extracted into diethyl ether after acidification of the media. Both activities could be recovered from a silicic acid column following elution with ethyl acetate and >95% of the biological activity was recovered in a single UV absorbent peak following HPLC (see Fig. 1). Identical results were obtained with rat PMNs. This peak had a retention time

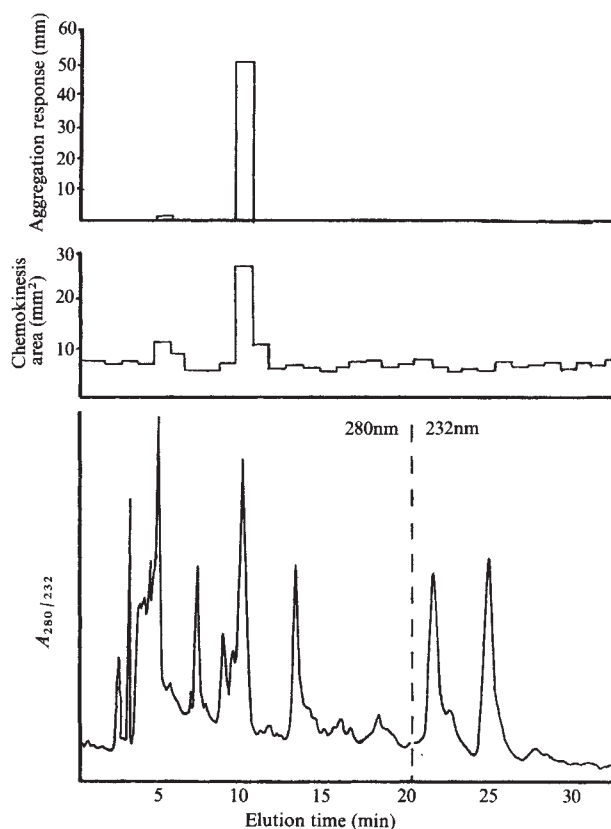


Fig. 1 Separation by HPLC of lipoxygenase products released by human PMNs exposed to $10\text{ }\mu\text{M}$ ionophore A23187 for 4 min as previously described⁵. The reaction was terminated by the addition of 1.5 vol. methanol, the cells were removed by centrifugation and the supernatant was acidified to pH 3.5 and extracted twice with diethyl ether. The ether extracts were washed once with water and dried under vacuum. The residue was dissolved in diethyl ether/hexane (20:80) and applied to a silicic acid column equilibrated with the same solvent¹. The column was subsequently eluted with ethyl acetate, the ethyl acetate was removed under vacuum and the residue dissolved in $50\text{ }\mu\text{l}$ methanol for application to a reverse phase HPLC column ($4.6 \times 250\text{ mm}$ packed with Spherisorb 50DS eluted at a flow rate of 1 ml min^{-1} with a mixture of methanol/water (75:25, v/v) plus acetic acid (0.01%)¹. HPLC columns were calibrated with a mixture of dimethylphthalate, di-*n*-butylphthalate and pyrene using as a solvent methanol/water (85:15, v/v). The elution times for 15-, 12- and 5-HETE were 24, 26.5 and 31.5 min, respectively. HPLC fractions were dried under vacuum and resuspended in medium for assay as below. Chemokinetic activity towards human PMNs was assayed in an agarose microdroplet assay¹¹, the results being expressed as the increase in the area of migration when compared with that of cells in the presence of medium alone. Aggregation of rat PMNs was assessed by nephelometry in a Payton aggregometer, the results being expressed as the change in transmittance as measured (in mm) on the recorder¹².

identical to that previously reported for leukotriene B (5, 12-dihydroxy 6, 8, 10, 14-eicosatetraenoic acid)¹. Ultraviolet spectra were obtained for the methyl ester of the compound which showed three major bands ($\epsilon_{\text{max}}^{\text{MeOH}}$ 262 ± 2 , 271 ± 2 and $282 \pm 2\text{ nm}$), indicating the presence of three conjugated double bonds⁶. Gas-liquid chromatographic analysis of the active fraction showed a major peak with a *C* value of 23.6. The mass spectrum of this compound is shown in Fig. 2 and the substance was identified as leukotriene B by comparison with the spectra previously reported for this compound⁷.

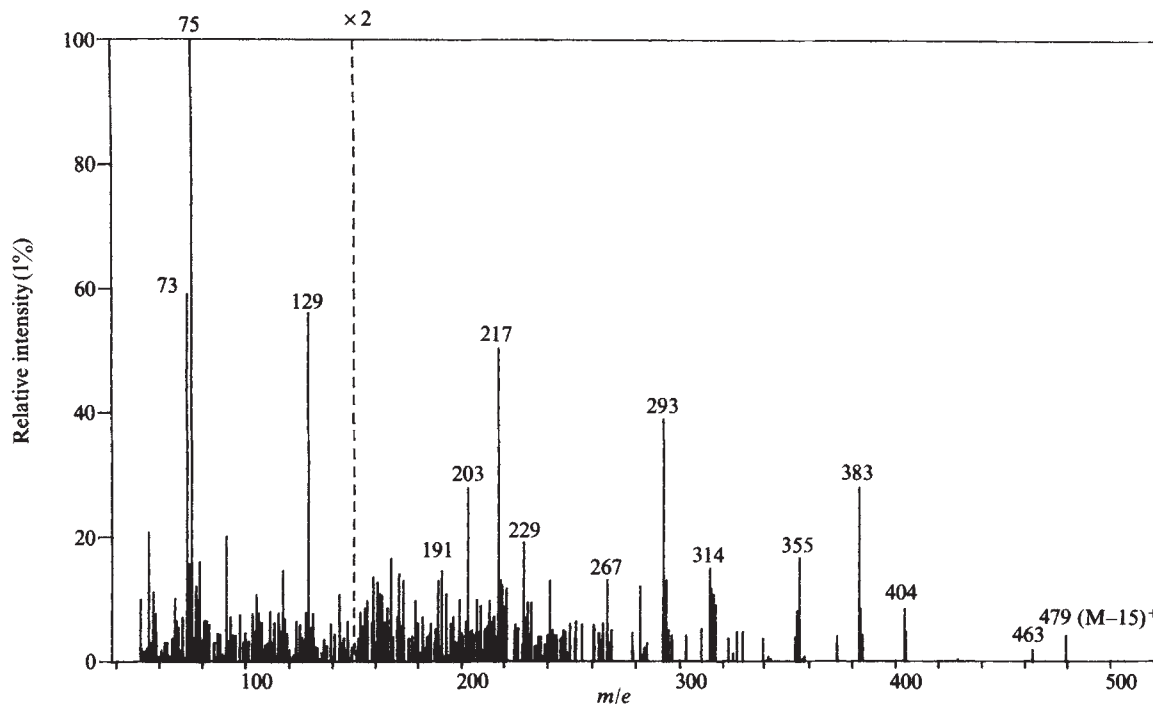


Fig. 2 Mass spectrum of the trimethylsilyl derivative of the methylester of leukotriene B obtained from rat peritoneal PMNs after incubation with A23187. The active fraction obtained from the HPLC column was methylated with diazomethane and further derivatized to the trimethylsilyl ether with bis (trimethylsilyl) trifluoroacetamide. The sample was chromatographed on a 20m SE54 WCOT column coupled to a VG Micromass 16F mass spectrometer. Spectra were put on to a data system in the following conditions; electron energy 70 eV, trap current 180 μ A and source temperature 240 $^{\circ}$ C. Peaks at m/e 281 and 355 represent bleed peaks from the column.

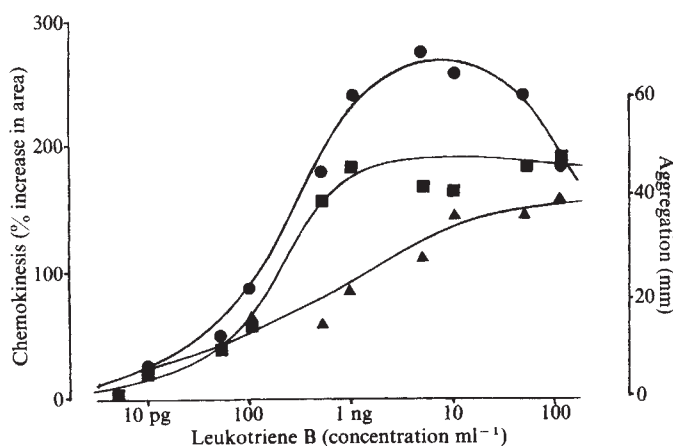


Fig. 3 The effects of leukotriene B on the chemokinesis of human PMNs (●) and the aggregation of rat (■) and human (▲) PMNs. Leukotriene B was prepared from 10^9 rat peritoneal PMNs incubated with calcium ionophore A23187 and was purified by ether extraction, silicic acid chromatography and HPLC and tested in aggregation and chemokinesis assays as described in Fig. 1 legend. The concentration of leukotriene B was determined using an ϵ_{281}^{MeOH} of 39,500 (ref. 6), 9 μ g being recovered from 10^9 cells. Each point represents two separate experiments carried out on separate days ($n=8-16$, standard errors were always $<10\%$ of the mean).

Figure 3 shows the effects of leukotriene B on the chemokinesis of human PMNs and the aggregation of rat and human PMNs. Dose-related effects were seen over the range 10 pg ml^{-1} to 5 ng ml^{-1} , ED_{50} values for human PMN chemokinesis, human PMN aggregation and rat PMN aggregation being 250, 560 and 170 pg ml^{-1} , respectively.

These results show that leukotriene B exhibits comparable activity on a molar basis to that observed for the complement peptide C5a and the synthetic peptide fMet-Leu-Phe. Furthermore, leukotriene B seems to be 100–1,000 times more active as a chemokinetic agent than the mono-HETEs^{3,8} and in the present experiments no aggregating or chemokinetic activity was recovered in the fractions where the mono-HETEs would be expected to elute.

Release of lipoxygenase products, including leukotriene B, may be a common feature of exposure of PMNs to a variety of inflammatory stimuli^{2,4}. Such release may be associated with the O_2 uptake, O_2^- production and stimulation of hexose monophosphate shunt activity that occur following exposure to such stimuli⁹ and may also be involved in PMN degranulation¹⁰. The much greater potency of leukotriene B, compared with that reported for the mono-HETEs, suggests that the latter compounds are of less biological significance. It will be of some interest to study the role of leukotriene B *in vivo* with respect to the accumulation of inflammatory cells at sites of inflammatory and allergic reactions.

We thank the Arthritis and Rheumatism Council (M.A.B.), the Science Research Council (M.V.D.) and Lilly Research Ltd for financial support.

Received 29 February; accepted 29 April 1980.

- Borgeat, P. & Samuelsson, B. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2148–2152 (1979).
- Stenson, W. I. & Parker, C. W. *J. clin. Invest.* **64**, 1457–1465 (1979).
- Goetzl, E. J. & Sun, F. F. *J. exp. Med.* **150**, 406–411 (1979).
- Goetzl, E. J., Weller, P. F. & Sun, F. F. *J. Immun.* **124**, 926–933 (1980).
- Bray, M. A., Ford-Hutchinson, A. W., Shipley, M. E. & Smith, M. J. H. *Br. J. Pharmac.* (in the press).
- Borgeat, P. & Samuelsson, B. *J. biol. Chem.* **254**, 7865–7869 (1979).
- Borgeat, P. & Samuelsson, B. *J. biol. Chem.* **254**, 2643–2646 (1979).
- Palmer, R. J., Stepney, R., Higgs, G. & Eakins, K. E. *Prostaglandins* (submitted).
- Bokoeh, G. M. & Reed, P. W. *Biochem. biophys. Res. Commun.* **90**, 481–487 (1979).
- Smolen, J. & Weissman, G. *Advances in Prostaglandin and Thromboxane Research* (Raven, New York, in the press).
- Smith, M. J. H. & Walker, J. R. *Br. J. Pharmac.* (in the press).
- Cunningham, F. M., Shipley, M. E. & Smith, M. J. H. *J. Pharm. Pharmac.* **32**, 377–380 (1980).

Identification of an interferon in murine placentas

Arnold K. Fowler*, Carl D. Reed* & David J. Giron†

*Carcinogenesis Intramural Program, National Cancer Institute, Frederick Cancer Research Center, Frederick, Maryland 21701

†Department of Microbiology and Immunology, Wright State University, School of Medicine, Dayton, Ohio 45435

It is becoming increasingly apparent that interferon exerts a major role in cellular regulatory processes. Besides its well known antiviral properties¹, interferon has been shown to affect cell surface antigen expression^{2,3}, lymphoid cell function^{4,5}, as well as cellular protein synthesis^{6,7} and growth⁸⁻¹¹. To date, however, there is little information available on the mechanism of interferon production *in vivo* except in response to a variety of exogenously administered viral and nonviral systemic inducers. We report here the detection of a pregnancy-associated elevation of interferon, or an interferon-like component, in selected reproductive tissues of mice.

Ten-week-old virgin NIH Swiss mice, a random-bred strain, were killed at specific times after mating. Female mice were examined daily and mating was indicated by the appearance of a copulation plug and was referenced as day 1 of gestation. Mice were maintained on a 12 h light cycle at a temperature of 24°C. Lab chow and water were available *ad libitum*. At autopsy the uteri were aseptically excised, carefully trimmed and the conceptuses removed. The conceptuses of individual mice were then pooled and after the 10-day stage dissected into placental and embryonic/fetal components. Earlier conceptuses (7-day stage) from individual mice were also pooled but were not dissected further. Immediately after removal, a 20% (w/v) homogenate of each specimen was prepared in phosphate-buffered saline (pH 7.2) and clarified for 30 min at 2,500g. Following storage at -76°C the samples were defrosted and the level of antiviral activity was determined by the 50% plaque reduction (PR₅₀) method as described previously¹²; see legend to Fig. 1. The titre of antiviral activity is expressed as the reciprocal of the highest dilution that inhibited plaque formation by 50%. One unit of antiviral activity is equivalent to 0.5 NIH (G002-904-511) reference units.

Preliminary studies showed the presence of an antiviral activity in mouse placental extracts. Biological and biophysical characterization of this activity as an interferon was determined by its protection of L-cell cultures against the cytopathic effects (CPE) of MM virus and is summarized in Table 1. The antiviral activity was sensitive to treatment with trypsin, actinomycin D, and heat and was neutralized by anti-interferon γ globulin. Also, it was not inactivated by low pH treatment (at pH 2) and was not removed by high speed centrifugation. By these criteria, the placental component exhibits the properties of virally induced, Type 1, interferon^{13,14}. It seems highly unlikely that the inhibition of CPE resulted from a nonspecific mechanism, such as an interference of virus attachment to the target L-cells by the tissue extracts, as the antiviral activity of the placental component requires a transcriptional event (sensitive to actinomycin D) and furthermore, is neutralized by specific anti-interferon γ globulin.

The interferon levels of uterine, placental and fetal tissues at various stages of gestation are shown in Fig. 1. As may be noted, interferon was detected in most uterine samples between 10-15 days of gestation; however, specimens taken before and after this interval of pregnancy, as well as from virgin mice ($n=3$, data not shown), had no detectable titre (<10 units ml⁻¹). This transient appearance of interferon in the gravid uterus developed abruptly and was highest at the 13-day stage

(300 units ml⁻¹). By comparison, interferon was found in all placental samples examined, and its level increased significantly during pregnancy. This increase was most dramatic between 10- and 13-day stages (8-fold) but continued until the 17-day stage. Subsequently, the interferon level generally plateaued at approximately 2,000 units ml⁻¹ until term. In other data not shown, the mean interferon level of 7-day old conceptuses from three individual mice was found to be essentially the same as 10-day placentas (110 as compared to 120 units ml⁻¹) and approximately 3-fold greater than 10-day fetuses (33 units ml⁻¹; right panel). Note that no fetal specimen had an interferon level greater than 80 units ml⁻¹ and further, that interferon-positive fetal samples were clustered around the 10-day stage, a stage at which contamination by extra-embryonic tissues is difficult to avoid. After this stage only one out of 6 fetal samples had a detectable interferon level.

These results clearly demonstrate a progressive increase in the level of interferon in the mouse placenta during gestation. Significant levels were detected in this organ as early as 10 days after mating, and most likely, the interferon detected in the 7-day conceptus was largely of placental origin. In contrast, little if any interferon was found in embryonic/fetal tissues. This observation is consistent with earlier reports that the placenta forms an effective barrier limiting migration of interferon into fetal circulation^{15,16}. Although a decrease in the efficacy of the placental barrier to interferon migration has been reported in the pregnant rat near term¹⁷, we have found no evidence of transplacental transfer of interferon into mouse fetuses in late gestation. This discrepancy may be attributed either to species differences or to the stage of gestation as we have not examined fetuses beyond the 18-day stage. The presence of an interferon antagonist in mouse embryos¹⁸ may also contribute to the apparently low levels of interferon in the embryo.

The significance of the transitory appearance of interferon in the uterus is more difficult to interpret. Although the level of interferon seems to be sufficiently elevated to indicate an active production or an accumulation process, we have not ruled out the possibility that uterine contamination by placental material

Table 1 Characterization of antiviral activity as interferon*

Treatment†	24 h CPE‡
None	0
pH 2.0	0
Ultracentrifugation	0
Trypsin	4†
Actinomycin D	4†
Heat	4†
Anti-IF γ globulin	4†
Virus control	4†
Cells controls§	0

*Placental extract containing 3,000 PR₅₀ units of antiviral activity diluted 1:10 in Dulbecco's MEM was used. A 1:100 dilution of this sample completely protected L-cell cultures against the cytopathic effects (CPE) of MM virus.

†pH adjusted to 2.0 with 1 M HCl; after 24 h at 4°C, pH adjusted to 7.0 with 1 M NaOH. Ultracentrifugation, Sample centrifuged at 100,000g for 2 h; supernatant fluid collected and assayed for antiviral activity. Trypsin, 0.25% trypsin for 30 min at 37°C. Actinomycin D, L-cell monolayer cultures were treated with Actinomycin D (0.5 μ g ml⁻¹) for 1 h before assay of the sample for antiviral activity. Heat, The sample was incubated at 56°C for 4 h. Anti-interferon γ globulin: The sample was incubated with an equal volume of anti-IF γ globulin (30) for 30 min at 37°C.

‡L-cell monolayer cultures were treated in duplicate for 4 h with each preparation. All the cultures, with the exception of cell controls, were then challenged with MM virus (about 10 p.f.u. per cell) and scored for CPE 24 h later. 0 = No CPE; 4+ = 100% CPE.

§Each preparation had its own cell control which was treated identically to the test sample but was not infected. Cell controls were all CPE negative.

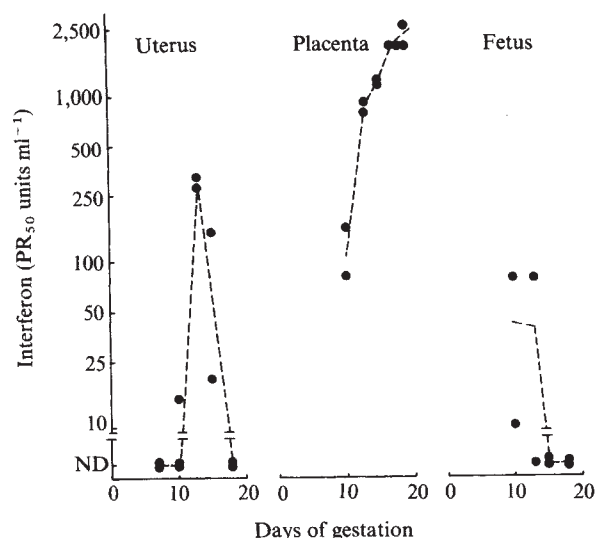


Fig. 1 Interferon levels of uterine, placental, and fetal tissues from NIH Swiss mice at various stages of gestation. For assay, twofold dilutions of each sample were made in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Each dilution was added to duplicate monolayer cultures of L929 cells. After 18 h at 37°C, the cultures were washed and challenged with approximately 100 plaque forming units (p.f.u.) of encephalomyocarditis virus (strain MM) and incubated for 48 h. The cultures were then stained and the plaques counted. Each point represents the antiviral activity of the tissue from an individual pregnant mouse.

is the source of the observed interferon. However, of the three explanations, the last seems least probable since a contamination of major proportion (>33%) would be necessary to account for uterine interferon level at the 13-day stage (300 units ml⁻¹). On the other hand, the production or accumulation of interferon in the gravid uterus, specifically between 10 and 15 days of gestation, would suggest stringent regulation.

The inductive event(s) of interferon production in the pregnant mouse and the mechanism of its concentration in the placenta are not known; however, studies in progress indicate that the maximum level of placental interferon in mice is under genetic control. We have not so far detected interferon in the serum (<10 units ml⁻¹) of either virgin or pregnant NIH Swiss mice. Whereas this may be interpreted as evidence favouring the *de novo* synthesis of interferon by the placenta, it does not preclude the possibility that this organ possesses an extraordinary capacity to concentrate interferon from maternal systemic pools. In the latter situation, induction by chronic parasitic infections in the mouse population could provide such a source of interferon. In our experiments, however, we have also examined tissues from specific pathogen-free NIH Swiss mice and in all cases, placental interferon levels of barrier-maintained mice were similar to mice housed in our conventional facility.

The demonstration that interferon accumulates in mouse placenta is interesting from several points of view. The placenta is an organ which occupies the intermediate zone between the maternal and fetal organisms. It is an organ of protective, endocrine, and immunological functions and is considered intricately involved in fetal acceptance¹⁹. Although a role for interferon in any of these events is conjectural, this class of glycoproteins exhibit numerous biological properties that are compatible with those of successful gestation. In addition to its viral prophylactic activity, interferon influences a variety of other physiological processes ranging from cell surface and growth control to immunoregulation. In this regard it is particularly noteworthy that prolongation of graft survival across H-2 barriers results with systemic interferon

inducers^{20,21}, as well as with the direct administration of interferon²¹⁻²³. Likewise, cell-mediated immunity in both graft-versus-host²⁴ and delayed-type hypersensitivity^{25,26} reactions was reduced by interferon treatment. These results and the findings that interferon also inhibits DNA synthesis induced in mouse lymphocytes *in vitro* by mitogens and allogeneic cells²⁷⁻²⁹ suggest a possible involvement of interferon in the immunoregulation of fetal acceptance. As we have also detected an antiviral activity in a human term placenta, the presence of interferon in this organ may not be unique to the murine species.

Received 26 November 1979; accepted 18 April 1980.

1. Isaacs, A. & Lindenmann, J. *Proc. R. Soc. B* **147**, 258-267 (1957).
2. Lindahl, P., Leary, P. & Gresser, I. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2785-2788 (1973).
3. Lindahl, P., Gresser, I., Leary, P. & Tovey, M. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1284-1287 (1976).
4. Johnson, H. M. & Baron, S. *CRC Critical Reviews in Biochemistry* **4**, 203-227 (1976).
5. Johnson, H. M. & Baron, S. *Pharmac. Ther.* **A1**, 349-367 (1977).
6. Brouty-Boye, D., Macieira-Coelho, A., Fiszman, M. & Gresser, I. *Int. J. Cancer* **12**, 250-258 (1973).
7. Fuse, A. & Kuwata, T. *J. gen. Virol.* **33**, 17-24 (1976).
8. Paucker, K., Cantell, K. & Henle, W. *Virology* **17**, 324-334, 1962.
9. Gresser, I. *et al. Proc. Soc. exp. Biol. Med.* **142**, 7-10 (1973).
10. Dahl, H. & Degre, M. *Acta path. microbiol. scand.* **84**, 285-292 (1976).
11. Stewart II, W. E., Gresser, I., Tovey, M. G., Bandu, M.-T. & LeGoff, S. *Nature* **262**, 300-302 (1976).
12. Giron, D. J., Allen, P. T., Pindak, F. F. & Schmidt, J. *Appl. Microbiol.* **21**, 387-393 (1971).
13. Giron, D. *Appl. Microbiol.* **18**, 584-588 (1969).
14. Youngner, J. S. & Salvin, S. B. *J. Immun.* **111**, 1914-1922 (1973).
15. Ho, M., Postic, B. & Yang, H. Ke. in *The Systemic Induction of IF*, in: *CIBA Foundation Symposium on IF* (eds Wolstenholme, G. E. W. *et al.* 19-35 (Churchill, London, 1967).
16. Overall Jr., J. C. & Glasgo, L. A. *Science* **167**, 1139-1141 (1970).
17. Mendelson, J., Kapusta, R. & Dick, V. *Am. J. Obstet. Gynec.* **107**, 902-907 (1970).
18. Cembrzynska-Nowak, M. *Archs Immun. Ther. Exp.* **25**, 663-668 (1977).
19. Jones, B. M. & Kemp, R. B. *Nature* **221**, 829-831 (1969).
20. Mobraaten, L. E., DeMaeyer, E. & DeMaeyer-Guignard, J. *Transplantation* **16**, 415-420 (1973).
21. Cerottini, J.-C., Brunner, K. T., Lindahl, P. & Gresser, I. *Nature new Biol.* **242**, 152-153 (1973).
22. DeMaeyer, E., Mobraaten, L. & DeMaeyer-Guignard, J. *C. r. hebdom. Séanc. Acad. Sci., Paris* **277**, 2101-2103 (1973).
23. Hirsch, M. S., Ellis, D. A., Black, P. H., Monaco, A. P. & Wood, M. L. *Transplantation* **17**, 234-236 (1974).
24. Hirsch, M. S., Ellis, D. A., Proffitt, M. R. & Black, P. H. *Nature new Biol.* **244**, 102-103 (1973).
25. DeMaeyer-Guignard, J., Cachard, A. & DeMaeyer, E. *Science* **190**, 574-576 (1975).
26. DeMaeyer, E., DeMaeyer-Guignard, J. & Vandeputte, M. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1753-1757 (1975).
27. Lindahl, P., Magnusson, P., Leary, P. & Gresser, I. *Nature new Biol.* **237**, 120-121 (1972).
28. Rozec, K. R., Lee, S. H. S. & Ngan, J. *Nature new Biol.* **245**, 16-18 (1973).
29. Heron, I., Berg, K. & Cantell, K. *J. Immun.* **117**, 1370-1373 (1976).
30. Giron, D. J., Liu, R. Y., Hemphill, F. E., Pindak, F. F. & Schmidt, J. P. *Proc. Soc. exp. Biol. Med.* **163**, 146-150 (1980).

Detection of a cell-surface antigen correlated with organ-specific metastasis

P. J. Shearman, W. M. Gallatin & B. M. Longenecker

Department of Immunology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Despite the fact that tumour cells with the potential for metastasis may circulate randomly, many demonstrate a preference for specific organs. Recently, several investigators have selected variant tumour cell lines with enhanced capacity to metastasize to specific organs, mainly using the spontaneously originating B16 melanoma cell line of the mouse and tumour variants with enhanced capacity to metastasize to the lungs¹, brain² and liver³. We previously reported^{4,5} the derivation of a liver-specific metastatic variant of a Marek's disease (MD) virus-transformed, non-producer^{4,6} lymphoma cell line. MD is a naturally occurring, herpes virus-induced, T-cell lymphoma of chickens which bears pathological and aetiological similarities to Burkitt's lymphoma in man. This makes MD a useful model for study⁷. One similarity is the pattern of metastasis in which both lymphomas induce a high incidence of ovarian and liver lesions^{8,9}. We now report the existence of a cell-surface antigen, detectable by a monoclonal antibody, correlated with organ-specific metastasis.

To quantify liver-specific metastasis in our system, we inject tumour cells intravenously into day 11 chick embryos and enumerate metastatic liver foci which form during 6 days of further incubation⁴. MDCC-AL2, a cell line which has been selected for liver-specific metastasis, forms many more embryonic liver foci than MDCC-RP1 or MDCC-AL1, lines not selected for organ-specific metastasis, or MDCC-AL3, selected for metastasis to the ovary⁴. Following the same *in ovo* procedure, cell lines which have been selected for increased virulence (RP1 < AL1 < AL2 < AL3) form increasingly more tumour foci on the chorioallantoic membrane (CAM) of the chick embryo. As these embryo assays are sensitive and convenient, we used them in inhibition assays to attempt to detect specific cell-surface antigens expressed on our liver-metastasizing, variant cell line, AL2.

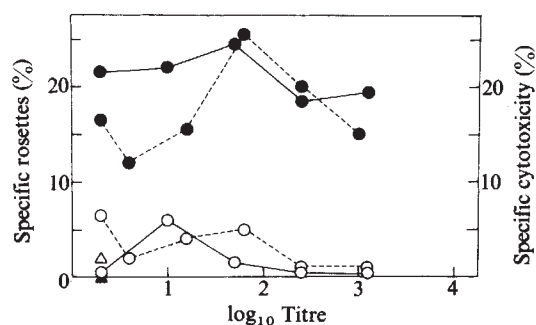


Fig. 1 Indirect rosettes (solid lines) were formed by incubating dilutions of hybridoma 1.20 culture supernatant with MDCC-AL2 cells (●) or MDCC-AL3 cells (○) for 60 min at 4°C. After three washes in culture medium, chicken red blood cells with rabbit anti-mouse immunoglobulin antiserum (Cappel) coupled to their surfaces by a modified 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma) technique were added at a 10:1 red cell-to-target cell ratio and incubated for 180 min at 4°C. Specific rosettes were calculated by subtracting background rosettes formed without culture supernatant. Complement-mediated cytotoxicity (broken lines) was accomplished by incubating RP1 (▲), AL1 (△), AL2 (●) or AL3 (○) cells with dilutions of hybridoma 1.20 culture supernatant and guinea pig complement for 45 min at 37°C. The cells were washed once in culture medium and cytotoxicity determined by dye exclusion. Specific cytotoxicity (broken lines) was calculated by subtracting cytotoxicity due to complement alone. In both assays, each point represents the average of two determinations.

For the generation of anti-tumour cell hybridomas, five CBA/J mice were injected intravenously with 10^8 AL2 cells. Three days later the spleen cells from the mice were pooled and fused with 315.43 myeloma cells as described previously¹⁰. Hybrid clones exhibiting positive direct or indirect agglutination activity in their culture supernatants against AL2 cells were picked and grown for further analysis. Sixteen clones proved to be stable and antibody-producers. Of these, nine clones seemed to detect chicken major histocompatibility complex (MHC, B locus) antigens. Only one clone produced antibody which reacted with all types of chicken cells tested, normal and malignant, and was considered to be anti-chicken panspecific. One clone produced antibody reacting with all lymphocytes tested regardless of MHC type. Four clones produced antibody which reacted with RP1, AL1, AL2, AL3 and another MD-derived lymphoma cell line, MDCC-MSB1, but which did not react with normal lymphocytes or erythrocytes. One clone, 1.20, produced antibody which reacted specifically with AL2 cells (Fig. 1) and showed little or no reactivity against chicken red or white blood cells of various genotypes, or tumour cells of the other MD-tumour cell lines.

To test whether the 1.20 antigen might be associated with specific liver metastasis, we measured the capacity of 1.20 antibody to inhibit liver foci induced by AL2 cells in the *in ovo* assay⁴. It was found that preincubation of AL2 cells with 1.20 monoclonal antibodies (Table 1) specifically blocks the ability of AL2 cells to colonize the embryonic liver without affecting the ability of the same cells to produce CAM foci. In contrast, CH-4 monoclonal antibodies which detect a chicken MHC antigen¹⁰ not found on AL2 cells, or 1.24 monoclonal antibodies which detect a public MHC antigen common to all MDCC-RP1-derived cell lines tested, inhibited neither CAM nor liver foci. To test another monoclonal antibody binding to AL2 cells, the capacity of 1.20 antibodies to inhibit liver foci was compared with that of 1.5 monoclonal antibodies, similar in specificity to 1.24 antibodies. This test (Table 2) was done with or without the prior complement-mediated killing of AL2 cells. Hybridoma 1.20 but not 1.5 monoclonal antibodies significantly inhibited the development of liver foci in the presence of complement. It is noteworthy that 1.5 monoclonal antibodies, which directly agglutinate the AL2 cells, caused a significant enhancement in the number of CAM foci induced by AL2 cells, suggesting that large emboli of tumour cells might be nonspecifically trapped in the CAM. In the same conditions, however, 1.5 monoclonal antibodies did not cause a significant enhancement in liver foci; this is consistent with our previous observations⁴ which suggest that homing of AL2 cells to the embryonic liver is a specific process and not simply a nonspecific trapping of emboli of tumour cells. The fact that 1.5 monoclonal antibodies caused a slight, but not significant, reduction in the number of liver foci suggests that at least a few of the liver focus-forming cells may have been trapped within cell aggregates. As different classes of mouse antibody may affect the homing of metastatic tumour cells differentially, we have standardized the class of antibody used. All the monoclonal antibodies used in our studies are IgM (unpublished data). This is in keeping with our previous observations¹⁰ which show that the mouse preferentially responds to chicken MHC antigens with antibodies of the IgM class in the same conditions with which the hybridomas were produced for the present study.

Two different *in vitro* assays, indirect rosette formation and complement-mediated cytotoxicity, were used to verify the specificity of 1.20-derived monoclonal antibodies. Both of these assays showed that approximately 20% of AL2 cells have detectable cell-surface antigen (Fig. 1). AL3 cells demonstrated a small but significant number of 1.20 antigen-positive cells.

To determine if the frequency of 1.20 antigen expression in a cell line correlates with the degree of liver metastasis exhibited by the cell line, we derived limiting dilution clones of metastatic variants AL2 and AL3. Six clones were isolated from AL2 and four from AL3. All 10 clones are non-producers for virus, tested as for the parent cell lines (data not

Table 1 Specific inhibition of liver metastasis by monoclonal antibody

Monoclonal antibody added	CAM foci	Liver foci
None	60 ± 17 (8)	63 ± 11 (8)
CH-4	59 ± 16 (8)	78 ± 21 (5)
1.20	61 ± 11 (7)	32 ± 10 (9)
1.24	68 ± 17 (10)	56 ± 6 (8)

Assays for chorioallantoic membrane (CAM) and embryonic liver foci were performed as described previously⁴. For CAM foci, 1×10^3 MDCC-AL2 cells were injected intravenously per embryo, and for liver foci, 3×10^4 AL2 cells were used. Before injection, cells were left untreated or preincubated for 30 min with ascites antibodies from hybridomas CH-4, 1.20 or 1.24 at a final dilution of 1:100. Results are expressed as means ± s.d. with number of embryos per group in parentheses. Statistically significant differences were obtained in comparing results in liver foci for 1.20 with controls ($P < 0.001$); 1.20 with CH-4 ($P < 0.01$) and 1.20 with 1.24 ($P < 0.001$).

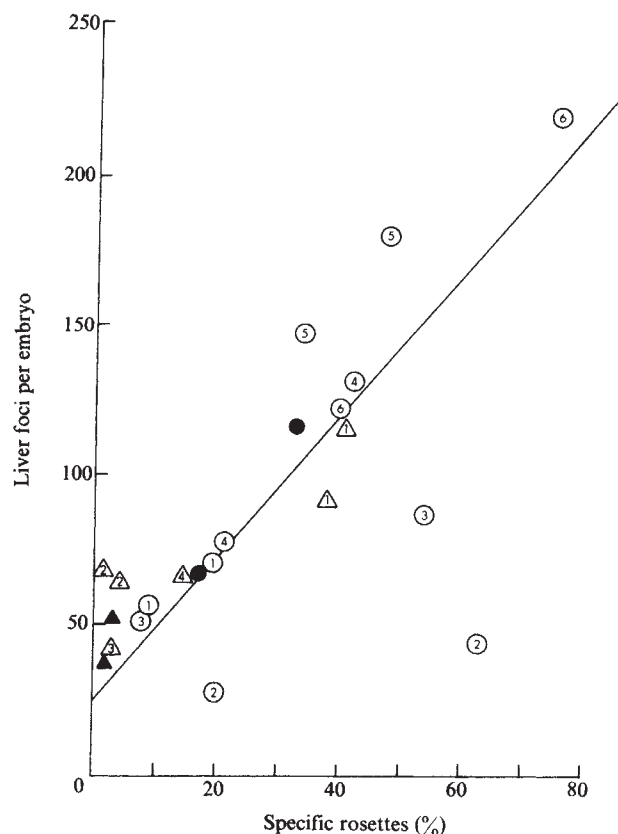


Fig. 2 The cell lines MDCC-AL2 (●) plus six limiting dilution clones (numbers in circle) and MDCC-AL3 (▲) plus four clones (numbers in triangles) were tested for 1.20 antigen frequency by the indirect rosette assay⁴ and for liver metastatic ability by the liver focus-forming assay⁴ on two occasions. Indirect rosettes were formed as described in Fig. 1 using a 1.20 antibody dilution of 1:100. A cell dose of 3×10^5 cells per embryo was used in the liver focus-forming assay. Paired values of 1.20 antigen frequency and average number of liver foci per embryo are plotted for each cell line and clone. These values demonstrate a correlation coefficient of 0.70 ($P < 0.001$).

Table 2 Specific inhibition of liver metastasis by monoclonal antibody plus complement

Monoclonal antibody added	CAM foci	Liver foci
None	70 ± 51 (5)	88 ± 14 (6)
C'	60 ± 38 (7)	75 ± 11 (6)
1.20	115 ± 33 (7)	62 ± 23 (7)
1.20 + C'	93 ± 15 (7)	34 ± 12 (8)
1.5	184 ± 58 (7)	76 ± 11 (8)
1.5 + C'	86 ± 29 (6)	63 ± 9 (8)

Assays for CAM and embryonic liver foci were performed as described previously⁴. Before injection, AL2 cells were incubated for 45 min at 37°C with the culture supernatants (full strength) from hybridomas 1.20 or 1.5 in the presence or absence of guinea pig complement (C'). The cells were washed and the cell concentrations were readjusted so that 1×10^5 viable AL2 cells were injected per embryo for CAM foci and 1×10^5 viable AL2 cells were injected per embryo for embryonic liver foci. 1.20 plus complement killed ~20% of AL2 cells and 1.5 plus complement killed ~40% of AL2 cells at the concentrations used. Results are expressed as means ± s.d. with number of embryos per group in parentheses. Statistically significant differences were obtained in comparing results in CAM foci for control or 1.5 + C' with 1.5 alone ($P < 0.01$); also for results in liver foci for C' or 1.5 + C' with 1.20 + C' ($P < 0.001$); 1.20 and 1.20 + C' ($P < 0.02$); Control and 1.20 ($P < 0.03$).

shown)^{4,6,11}. According to the work of Fidler and co-workers¹², these clones should vary in their metastatic ability and possibly their antigenic profile. We tested each clone as well as the parent cell lines on two occasions for both indirect rosette-forming cells and liver focus-forming ability. Cell line AL2 and its clones ranged from 8 to 76% in frequency of 1.20 antigen-positive cells. AL3 and its clones ranged from 1 to 41%. For the liver focus assay, we injected a relatively large number of cells to allow maximal precision of the assay. The paired observations are plotted in Fig. 2 and show that the number of liver foci formed by a clone is directly proportional to the number of 1.20 antigen-positive cells in that clone ($r = 0.70$, $P < 0.001$). Clone AL3.1 demonstrates a high frequency of 1.20 antigen-positive cells and showed high liver metastatic ability on both occasions. In contrast, all the other AL3-derived clones have a low value for each of these parameters as does the uncloned parental line AL3. Note that there is some day-to-day variation in 1.20 antigen expression and liver focus-forming ability, although successive assays correlate very highly. Thus, although correlated and possibly associated in a functional sense, the parameters of antigen expression and metastasis may vary in the strength of the correlation between them for a given clone. This is not unexpected as metastasis is a complex series of events only one of which involves specific organ recognition. Kerbel¹³ has reported that random cloning produces unstable metastatic variants in contrast to the work of Fidler¹². Stable metastatic variants were produced only after treatment with mutagens and lectins¹³. In our studies, we have found a strong and relatively stable correlation between 1.20 antigen expression and liver metastasis. This strengthens the argument in conjunction with the 1.20 antibody-mediated inhibition of liver metastases that the 1.20 antigen is involved in determining liver-specific metastasis.

Thus, we have described the existence of a cell-surface antigen, detectable by a monoclonal antibody which is correlated with specific liver metastasis of a lymphoma cell line. Cell populations with a low capacity to form liver foci have a low expression of the 1.20 antigen specificity and cell populations with a high capacity to form liver foci have a high level of 1.20 antigen expression. Monoclonal antibodies directed against the 1.20 antigen inhibit specific liver metastasis by AL2 cells but do not inhibit CAM lesions, which is a measure of the invasiveness of the cell line⁴. Our working hypothesis is that the 1.20 antigen has a functional role in the interaction of a blood-borne, metastasizing tumour cell with the target organ, the liver. The metastatic process is a complex series of events¹² and our results with clone AL3.1 have shown that 1.20 antigen expression is a necessary but not a sufficient condition in the determination of successful liver metastases.

This work was supported by the NCI and MRC of Canada. B.M.L. is a Research Associate and W.M.G. is a Research Student of the NCI of Canada.

Received 4 December 1979; accepted 21 April 1980.

- Fidler, I. J. *Nature new Biol.* **242**, 148–149 (1973).
- Nicolson, G. L. & Brunson, K. W. *Gann* **20**, 15–24 (1978).
- Tao, T., Matler, A., Vogel, K. & Burger, M. M. *Int. J. Cancer* **23**, 854–857 (1979).
- Shearman, P. J. & Longenecker, B. M. *Int. J. Cancer* **25**, 363–369 (1980).
- Report of the Ad hoc Committee on Avian Cell Line and Transplantable Tumour Nomenclature *Avian Path.* **8**, 487–498 (1979).
- Nazerian, K. et al. *Avian Dis.* **21**, 69–76 (1976).
- Klein, G. in *Oncogenesis and Herpesvirus* (eds Briggs, P. M., de Thé, G. & Payne, L. N.) 501–515 (IARC, Lyons, 1972).
- Payne, L. N. in *Oncogenesis and Herpesvirus* (eds Briggs, P. M., de Thé, G. & Payne, L. N.) 21–37 (IARC, Lyons, 1972).
- Wright, D. H. in *Oncogenesis and Herpesvirus* (eds Briggs, P. M., de Thé, G. & Payne, L. N.) 217–229 (IARC, Lyons, 1972).
- Longenecker, B. M., Mosmann, T. R. & Shiozawa, C. *Immunogenetics* **9**, 137–148 (1979).
- Longenecker, B. M., Pazderka, F., Stone, H. S., Gavora, J. D. & Ruth, R. F. *Infect. Immun.* **11**, 922–931 (1975).
- Fidler, I. J., Gerstein, D. M. & Hart, I. R. *Adv. Cancer Res.* **28**, 149–250 (1978).
- Kerbel, R. S. *Am. J. Path.* **97**, 609–622 (1979).

T-cell hybridoma bearing heavy chain variable region determinants producing (T,G)-A--L-specific helper factor

Zelig Eshhar, Ron N. Apte, Ilana Löwy,
Yinon Ben-Neriah, David Givol & Edna Mozes

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

Thymus-derived lymphocytes (T cells) exert their regulatory effect (help or suppression) on the antibody production by B cells either by direct cell to cell interaction or by soluble mediators or factors^{1,2}. The low frequency of specific T cells, the heterogeneity of their responses and their relatively short life span have hampered the molecular characterization of the antigen recognition unit of T cells, and its structure is largely unknown. The lymphocyte hybridization technique, which has been found very useful for the production of B-cell hybridomas secreting specific monoclonal antibodies³, has also been used for the generation of homogeneous and stable T-cell hybridomas with unlimited growth potential^{4,5}. So far the only specific effector function demonstrated in the established T hybridomas is the property to generate a factor(s) which suppresses antibody responses⁶⁻⁸. We now describe the establishment of hybrid lines which exhibit characteristic T-cell markers. One of the hybridomas (denoted R-9) releases into the culture supernatant factor(s) with helper activity specific to the synthetic polypeptide (T,G)-A--L and bears surface determinants of the immunoglobulin heavy chain variable region (V_H). Such hybrid cell lines are of great value for studies on the nature of the T-cell receptor.

Cell fusion was carried out essentially as described previously⁹, using 41% polyethylene glycol 1500 (PEG) as fusing agent (Table 1). The AKR/J thymoma line BW5147, which lacks the enzyme hypoxanthine guanine phosphoribosyl transferase (derived by Dr R. Hyman), was fused with an enriched population of activated T cells. Activation of T cells to (T,G)-A--L was carried out by the transfer of thymocytes from C3H.SW (H-2^b) mice into irradiated (750 R) syngeneic recipients together with 10 µg of (T,G)-A--L (ref. 10). One week after transfer and 24 h before hybridization, 'educated' T cells from the spleens of the recipient mice were cultured on monolayers of (T,G)-A--L-bearing splenic adherent cells. This procedure augments the helper activity of (T,G)-A--L educated cells (R.N.A. and E.M., unpublished). After hybridization, cell suspensions were distributed into 24-well culture plates (Costar) and selected for 3 weeks in medium containing hypoxanthine, aminopterin and thymidine (HAT). Hybrid populations were grown in Dulbecco's modified Eagle's medium (DMEM) containing 15% horse serum (DMEM-HS). One month after fusion, 21 individual hybridoma cultures had been established.

The hybrids were screened for the presence of T-cell surface markers, immunoglobulin constant and variable (V_H and V_L) determinants¹¹ and (T,G)-A--L-specific biological activity. Most of the hybrid lines expressed the Thy 1.1 and Thy 1.2 alleles (originated from the parental BW5147 and C3H.SW lymphocytes, respectively). None of the hybridomas reacted with anti-V_L or anti-Ig antibodies, whereas 5 out of the 21 lines reacted with anti-V_H antibodies. Three of the V_H-positive hybrid lines—R-9, R-11 and R-17—affected the antibody response to (T,G)-A--L. R-11 demonstrated suppressive activity which will be described elsewhere. Here we present the characteristics of the R-9 hybrid line which exhibits antigen-specific helper activity.

Detailed analysis of the surface antigens (double immunofluorescence staining) and karyotype of R-9 hybridoma are shown in Table 1. The hybrid nature is indicated by the average chromosome number (65), which is higher than diploid (40), and by the expression of the H-2 and Thy-1 alloantigens of both parental strains on R-9 cells. Cytotoxicity assays using anti-Lyt reagents and rabbit complement resulted in 40% lysis of the R-9 cells with anti-Lyt-1.1, whereas no lysis was observed following treatment with anti-Lyt-2.1. Hybrids were not analysed for the Lyt-3.2 marker. The presence of immunoglobulin variable-region determinants was detected by using the F(ab')₂ of specifically purified rabbit antibodies against isolated V_H and V_L fragments derived from M-315 myeloma protein¹¹. Most of the R-9 cells did express V_H determinants whereas neither BW5147 cells nor R-19 (another hybrid line derived from the same fusion) reacted with anti-V_H antibodies. The distribution of stained cells was further analysed by the fluorescence-activated cell sorter (FACS-II) as depicted in Fig. 1. Whereas R-9 cells were labelled with anti-V_H antibodies in a manner which suggests a homogeneous population of the stained cells, they failed to react with either anti-V_L or anti-Ig antibodies. Computer analysis of the relative cell number stained with anti-V_H indicated that 70–85% of R-9 cells were positively labelled. BW5147 cells were not stained by either anti-V_H or anti-V_L antibodies (Fig. 1).

The antigen-specific activity of the hybrid lines was evaluated by their ability to produce a factor(s) with helper activity in the (T,G)-A--L-specific antibody response. Supernatants from 48–72-h cultures (containing 1–2 × 10⁶ hybrid cells per ml) were injected together with (T,G)-A--L and 2 × 10⁷ primed B cells into irradiated syngeneic recipients. Mice were bled 10–14 days later and (T,G)-A--L-specific antibodies in their sera were determined as shown in Table 2. Supernatants from R-9 and R-17 hybridomas induced anti-(T,G)-A--L responses when transferred together with T-cell-depleted primed spleen cells and (T,G)-A--L. Such activity was not manifested by supernatants of BW5147 cells and 19 other hybridomas of which R-19 is a representative. The degree of help provided by the active supernatants was not as high as with the T cells in the unfractionated primed spleen cells, but it was comparable to that of 5 × 10⁶ (T,G)-A--L-activated T

Table 1 Cell-surface markers and karyotype analysis of T hybridomas

Antisera or antibodies	BW5147	R-9	R-19
		% Stained cells	
Anti-Thy 1.1 (1:100)	>95	>95	>95
Anti-Thy 1.2 (1:100)	<5	>90	85
Anti-H-2 ^k (1:20)	>95	>95	>95
Anti-H-2 ^b (1:20)	<5	>95	>95
Anti-Ig (0.5 mg ml ⁻¹)	<5	<5	<5
Anti-V _H (0.3 mg ml ⁻¹)	<10	82	<10
Anti-V _L (λ) (0.3 mg ml ⁻¹)	<5	<10	<5
Anti-V _L (κ) (0.3 mg ml ⁻¹)	<5	<5	<5
Karyotype*	41(40–45)	65(60–72)	65(59–75)

Fusion was carried out by the addition of 41% PEG 1500 in serum-free DMEM to a cell pellet of 36 × 10⁶ enriched activated T cells from C3H.SW mice (H-2^b, Thy 1.2, Lyt 1.1, Lyt 2.1, Lyt 3.2) and 10 × 10⁶ AKR/J BW5147 thymoma. After 2 min at 37°C, PEG was gradually diluted with serum-free DMEM and after centrifugation the cells were suspended in DMEM-HS to a concentration of 10⁵ viable BW cells per ml. Aliquots (1 ml) were distributed into each well of 24-well culture plates. Medium containing HAT was added the next day and cultures were fed each third day with fresh HAT medium. The % of stained cells was analysed by indirect immunofluorescence using fluorescein-conjugated (FITC) rabbit antibodies against mouse IgG (Miles-Yeda) that were intensively absorbed with mouse T-cell lines and thymocytes for the detection of the murine alloantisera. FITC coupled to F(ab')₂ of goat anti-rabbit IgG was used for the detection of the rabbit antibodies against the murine variable region (V_H and V_L) and whole immunoglobulins (Ig). Anti-Thy 1.2 and anti-Thy 1.1 are monoclonal antibodies. Anti-H-2^k antiserum was obtained by the immunization of C3H.SW mice with C3H/DiSn lymphocytes and for the production of anti-H-2^b antiserum C3H/DiSn lymphocytes and for the production of anti-

*Chromosome number was detected 8 months after fusion. In each determination 10 mitotic cells were counted. The range of chromosome number is given in parentheses.

Table 2 (T,G)-A--L-specific helper activity of culture supernatants of T hybridoma R-9

Cells and supernatants transferred into irradiated recipients	Expt 1	Expt 2	Expt 3
	Antibody response to (T,G)-A--L (c.p.m.)		
2×10^7 (T,G)-A--L-primed spleen cells	12,185	9,777	13,562
2×10^7 (T,G)-A--L-primed B cells*	1,880	2,029	754
2×10^7 (T,G)-A--L-primed B cells + 5×10^6 (T,G)-A--L 'educated' T cells†	4,947	4,370	5,163
2×10^7 (T,G)-A--L-primed B cells + supernatant of BW5147	2,671	1,659	NT
2×10^7 (T,G)-A--L-primed B cells + supernatant of line R-19‡	NT	NT	1,508
2×10^7 (T,G)-A--L-primed B cells + supernatant of line R-17‡	5,125	NT	NT
2×10^7 (T,G)-A--L-primed B cells + supernatant of line R-9‡	4,164	4,619	4,380
2×10^7 (T,G)-A--L-primed B cells + R-9 eluate from a (T,G)-A--L-Sepharose column§	15,393	NT	NT
	Antibody response to ovalbumin (c.p.m.)		
2×10^7 Ovalbumin-primed spleen cells	11,257	9,843	
2×10^7 Ovalbumin-primed B cells*	4,521	2,523	
2×10^7 Ovalbumin-primed B cells + supernatant of line R-9	4,346	1,820	
2×10^7 Ovalbumin-primed B cells + supernatant of BW5147	4,054	NT	
2×10^7 Ovalbumin-primed B cells + supernatant of line R-17	7,008	NT	

Cells and supernatants were transferred into 750-R irradiated C3H.SW recipient mice together with 10 µg of (T,G)-A--L or 100 µg of ovalbumin. Antibody responses in antisera of mice bled 12–14 days after transfer were measured by plate radioimmunoassay; 0.05 mg ml⁻¹ of either (T,G)-A--L or ovalbumin diluted in phosphate-buffered saline PBS were used to coat the wells of flexible plates. Antigen-coated plates were incubated with aliquots of 1:20 dilution of pooled sera (5–10 mice per group). The plates were then incubated with ¹²⁵I-labelled goat anti-mouse Fab. Results are expressed as mean c.p.m. of triplicate wells. NT, Not tested.

*Anti-Thy 1.2 and complement-treated primed spleen cells.

†Educated T cells obtained from spleens of irradiated recipients 1 week after transfer of 10⁸ thymocytes and immunization with 10 µg (T,G)-A--L in complete Freund's adjuvant.

‡Supernatants (1 ml) of hybridoma cultures grown to a concentration of 1–2 × 10⁶ cells per ml were injected into each recipient mouse together with B cells and antigen.

§R-9 supernatant was passed through a (T,G)-A--L-Sepharose column. Elution was with 0.1 M NH₄OH. The dialysed eluate was injected at a twofold concentration of the original supernatant.

cells (Table 2). The antigen specificity of the help provided by the factor was analysed in a parallel system using ovalbumin-primed splenic lymphocytes. As shown in the lower section of Table 2, R-9 supernatants which contain helper factor for (T,G)-A--L failed to reconstitute the anti-ovalbumin response in recipients of ovalbumin-primed B lymphocytes. However, R-17 supernatants functioned as non-antigen-specific mediators and provided help both to (T,G)-A--L and ovalbumin responses.

A further confirmation for the (T,G)-A--L specificity of R-9 supernatants is provided by the high efficiency of an eluate of a (T,G)-A--L Sepharose column in replacing T cells in antibody production to (T,G)-A--L. As shown in Table 2 the antibody levels produced in recipients of (T,G)-A--L-primed B cells reconstituted with such an eluate (at a twofold concentration of the original supernatant) are comparable to those observed in recipients of primed spleen cells.

To determine whether the antigen-specific factor carries V_H determinants, as shown for R-9 hybrid cells, the above eluate of a (T,G)-A--L column was applied to immunoadsorbents made of anti-V_H and of anti-mouse immunoglobulin. The effluents and the 0.1 M NH₄OH eluates of these columns were analysed for their helper activity in an *in vitro* antibody production system¹² which enabled us to assay small doses of the factor. In this system nitro-iodophenyl (NIP)-ovalbumin primed spleen cells served as a source of hapten-specific B cells. The ability of the different R-9 fractions to provide help to these cells in the presence of NIP-(T,G)-A--L was determined by measuring the NIP-specific plaque forming cell (PFC) responses (Table 3). As shown in Table 3, the eluate of a (T,G)-A--L-Sepharose column provided (T,G)-A--L-specific helper activity; this was also shown *in vivo* in the transfer experiments (Table 2). In addition, as demonstrated in Table 3, the activity of the (T,G)-A--L-Sepharose eluate was removed by an anti-V_H-Sepharose column and could be eluted from this immunoadsorbent with 0.1 M NH₄OH. On the other hand, the helper activity could not be retained in an eluate of a guinea-pig anti-mouse immunoglobulin immunoadsorbent. Thus, the (T,G)-A--L-specific helper factor of R-9 hybridoma possesses the same V_H determinants as the cell line itself.

The R-9 hybrid line has expressed V_H determinants and produced active helper factors for 15 months since its establishment. This line could be cryopreserved and fully

retained its properties. R-9 cells have been further cloned on soft agar. All 45 clones analysed reacted with the anti-V_H antibodies. Preliminary screening revealed helper activity in about 50% of the clones.

Antigen-specific helper and suppressor T cells and their products have been reported to possess determinants encoded by the I region of the major histocompatibility complex^{13–18} and variable-region gene products^{19–23}. We have previously described the generation and partial characterization of a helper T-cell factor specific for the synthetic polypeptide (T,G)-A--L^{10,24}. The helper T-cell-replacing factor could be absorbed to immobilized antigen, anti-I-A antibodies and anti-idiotypes against (T,G)-A--L-specific antibodies^{13,14,22}. Similarly, factors with suppressor activity have been shown to possess both I-J and idiotype determinants^{16,17,23}. More general reagents of potential reactivity against the T-cell receptor are the anti-V-region antibodies directed against the common framework-related determinants (V_H)¹¹. Such anti-V_H antibodies effectively interfered with T-cell activities²⁵ and reacted with some T-cell products²⁶. In accordance, we report here the establishment of an immunoglobulin-negative V_H-positive T-cell hybrid line producing a (T,G)-A--L-specific helper factor which bears V_H determinants.

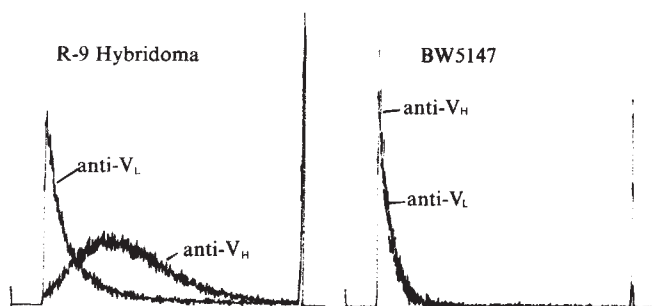


Fig. 1 Immunofluorescence profile of R-9 hybridoma and its parental BW5147 thymoma labelled with either anti-V_H or anti-V_L (λ) purified rabbit F(ab')₂ antibody fragments. Fluorescein conjugated F(ab')₂ of goat anti-rabbit IgG was used for staining. Abscissa, fluorescence intensity; ordinate, relative cell number.

Table 3 (T,G)-A--L-specific helper factor of R-9 hybrid line possesses V_H determinants

NIP-OVA primed spleen cells cultured with:	NIP specific PFC per 10 ⁶ viable cells*			
	NIP-(T,G)-A--L		NIP-FGG	
	Expt 1	Expt 2	Expt 1	Expt 2
(T,G)-A--L-primed spleen cells	279 ± 43	81 ± 23	175 ± 24	66 ± 28
Euate from (T,G)-A--L-Sepharose†	1,333 ± 76	500 ± 33	375 ± 25	47 ± 8
Effluent from anti-mouse Ig-Sepharose	1,045 ± 57	487 ± 23	213 ± 31	111 ± 17
Euate from anti-mouse Ig-Sepharose‡	416 ± 36	388 ± 71	196 ± 33	103 ± 38
Effluent from anti-V _H -Sepharose	230 ± 13	102 ± 31	114 ± 45	46 ± 26
Effluent from anti-V _H -Sepharose	175 ± 20	62 ± 32	151 ± 19	48 ± 17
Euate from anti-V _H -Sepharose‡	621 ± 58	413 ± 34	108 ± 24	73 ± 27

NIP-Ovalbumin primed spleen cells were cultured in microtitre plates (10⁶ per well) together with either (T,G)-A--L-primed spleen cells (2.5 × 10⁵ per well) or the different effluents and euates (final concentration 25%). Either NIP-(T,G)-A--L or NIP-FGG (fowl γ-globulin) (1 μg) was added to each well. Culture conditions were as described elsewhere¹².

*Indirect plaque forming cells (PFC) were determined using NIP-sheep red blood cell indicator cells. Results are expressed as the mean number of quadruplicates tested ± s.e. NIP-Ovalbumin primed spleen cells cultured in the presence of homologous antigen gave 1,794 PFC and 678 PFC whereas the background without antigen was 68 PFC and 50 PFC in Expts 1 and 2 respectively.

†R-9 eluted from (T,G)-A--L-Sepharose column (as described in Table 2 legend). This affinity-purified fraction was further passed through the rabbit anti-V_H and guinea pig anti-mouse IgG-Sepharose columns.

‡Elution was with 0.1 M NH₄OH.

Experiments are under way to characterize the products of R-9, the first T-cell hybridoma which is a constitutive producer of (T,G)-A--L-specific helper factor and bears surface variable-region determinants. The information obtained may clarify the nature of the molecular association between MHC-coded determinants and immunoglobulin variable-region gene products in the T-cell recognition unit.

We thank Ms Heidy Zinger and Ms Tova Waks for technical assistance, Dr Patrick de Baetselier for analysing the hybridomas in the FACS, and Drs R. Hyman, A. Peled and A. Rothstein for BW5147 thymoma, anti-Lyt 2.1 antibodies, and anti-Thy 1.2 and 1.1 antibodies, respectively. This research was supported in part by a grant from the National Council for Research and Development, Israel, and the DKFZ, Heidelberg, FRG. Z.E. is an incumbent of the Recanati Career Development Chair in Cancer Research. R.N.A. is a recipient of the Wolf Michaels postdoctoral fellowship.

Received 23 January; accepted 29 April 1980.

- Katz, D. H. & Benacerraf, B. *Adv. Immun.* **15**, 1-94 (1972).
- Tada, T. & Okumura, K. *Adv. Immun.* **28**, 1-87 (1980).
- Köhler, G. & Milstein, C. *Nature* **256**, 495-497 (1975).
- Goldsby, R. A., Osborne, B. A., Simpson, E. & Herzenberg, L. A. *Nature* **267**, 707-708 (1977).
- Hammerling, G. J. *Eur. J. Immun.* **7**, 743-746 (1977).
- Kontianen, S. *et al. Nature* **274**, 477-480 (1978).
- Taussig, M. J., Corvalan, J. R. F., Binns, R. M. & Holliman, A. *Nature* **277**, 305-308 (1979).
- Taniguchi, M., Saito, T. & Tada, T. *Nature* **278**, 555-558 (1979).
- Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W. & Howard, J. C. *Nature* **266**, 550-552 (1977).
- Taussig, M. J., Mozes, E. & Isac, R. *J. exp. Med.* **140**, 301-312 (1974).
- Ben-Neriah, Y., Lonai, P., Gavish, M. & Givol, D. *Eur. J. Immun.* **8**, 792-796 (1978).
- Mishell, R. I. & Dutton, R. W. *J. exp. Med.* **126**, 423-442 (1967).
- Taussig, M. J., Munro, A. J., Campbell, R., David, C. S. & Staines, N. A. *J. exp. Med.* **142**, 694-700 (1975).
- Isac, R., Dorf, M. E. & Mozes, E. *Immunogenetics* **5**, 467-475 (1977).
- Feldmann, M. *et al. in Ir Genes and Ia Antigens* (ed. McDavitt, H. O.) 315-323 (Academic, New York, 1978).
- Tada, T., Taniguchi, M. & David, C. S. *Cold Spring Harb. Symp. quant. Biol.* **41**, 119-127 (1977).
- Thöze, J., Kapp, J. A. & Benacerraf, B. *J. exp. Med.* **195**, 839-856 (1977).
- Okumura, K., Herzenberg, L. A., Murphy, D. B., McDavitt, H. O. & Herzenberg, L. A. *J. exp. Med.* **144**, 685-698 (1976).
- Binz, H. & Wiggall, H. J. *J. exp. Med.* **142**, 197-211 (1975).
- Eichmann, K. & Rajewsky, K. *Eur. J. Immun.* **5**, 661-666 (1975).
- Krawinkel, U., Cramer, M., Melchers, I., Imanishi-Kari, T. & Rajewsky, K. *J. exp. Med.* **147**, 1341-1347 (1978).
- Mozes, E. & Haimovich, J. *Nature* **276**, 56-57 (1979).
- Germain, R. N., Ju, S.-T., Kipps, T. J., Benacerraf, B. & Dorf, M. E. *J. exp. Med.* **149**, 613-622 (1979).
- Isac, R. & Mozes, E. *J. Immun.* **118**, 584-588 (1977).
- Eichmann, K., Ben-Neriah, Y., Givol, D. & Lonai, P. *Eur. J. Immun.* (in the press).
- Cramer, M. *et al. Eur. J. Immun.* **9**, 332-338 (1979).

Small subunit of I-A subregion antigens determines the allospecificity recognized by a monoclonal antibody

Jack Silver, Susan L. Swain* & Jeffrey J. Hubert

The Department of Cellular and Developmental Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, California 92037

*Department of Biology, University of California San Diego, La Jolla, California 92037

The murine major histocompatibility complex (MHC) codes for three groups of identifiable cell-surface proteins, the K, D molecules, the I-A subregion antigens and the I-E subregion antigens. All three groups of molecules display a high degree of serologically detectable polymorphism¹ and consist of two noncovalently associated polypeptides²⁻⁴. Amino acid sequence and peptide comparisons among allotypes of K, D and I-E molecules reveals that one polypeptide is relatively constant, whereas the other is highly variable⁹⁻¹³. Thus, it is likely that only one of the two polypeptides, the variable component, determines the antigenic specificities recognized by alloantisera. In contrast to the K, D and I-E molecules, both subunits of I-A molecules display substantial structural differences when comparisons among allotypes are made^{4,14-16}. Therefore, we have investigated whether one or both subunits of I-A molecules determine their alloantigenic specificities. Our results, presented here, indicate that only one of the two subunits determines a particular allospecificity recognized by a monoclonal antibody.

I-A molecules radiolabelled with ³H-phenylalanine were isolated from B10.A(5R) mice by indirect immunoprecipitation with (B10.A × A)F₁ anti-B10.A(5R) antisera¹⁴ (Fig. 1b). I-A molecules were isolated from B10.A(4R) and [B10.A(4R) × B10.A(5R)]F₁ mice with monoclonal antibody 10-2.16 (Fig. 1a,c), which is unreactive with B10.A(5R) mice and putatively directed against the public antigenic specificity Ia.17 based on its pattern of reactivity (Table 1 and ref. 17). The larger (A_β) and small (A_α) chains were purified by SDS-polyacrylamide gel

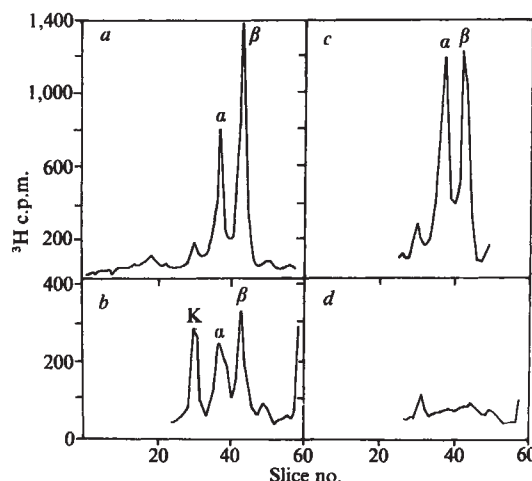


Fig. 1 SDS-PAGE profiles of I-A antigens immunoprecipitated from B10.A(4R) (a), B10.A(5R) (b) and [B10.A(4R) × B10.A(5R)]F₁ (c) mice. I-A antigens consist of two polypeptide chains designated α (molecular weight 37,000) and β (MW 28,000). The antiserum used to immunoprecipitate I-A antigens from B10.A(5R) mice is multispecific and also precipitates the H-2K molecule (MW 45,000). d Illustrates the inability of monoclonal antibody 10-2.16 to precipitate any I-A molecules from B10.A(5R) mice.

Table 1 Cytotoxic activity of reagent from 10-2.16

Spleen from:	I-A	I-E/C	Reciprocal titre
B10.BR	k	k	160
B10.D2	d	d	<10
B10.A	k	k/d	160
B10.A(4R)	k	b	160
B10	b	b	<10
B10.G	q	q	<10
B10.S	s	s	320
PL/J	u	u	160
B10.LPRIII	r	r	160
B10.M	f	f	160
B10.A(5R)	b	k/d	<10

Monoclonal antibody used to precipitate I-A subregion antigens from B10.A(4R) and [B10.A(4R) × B10.A(5R)]F₁ mice was produced by collecting and concentrating supernatants from cultures of hybridoma 10-2.16. This hybridoma was originally produced by fusing splenocytes from a CWB mouse immunized with C3H cells with the NS-1 myeloma. Cytotoxic activity was measured as described previously³². The resultant monoclonal antibody only lyses cells which possess the I-A^{k,s,f,r,u} subregion, and is putatively directed against the antigenic specificity Ia.17 based on this pattern of reactivity¹⁷.

electrophoresis (PAGE)¹⁸ and tryptic peptide maps were obtained as described elsewhere^{19,21}. A₂ chains isolated from B10.A(4R) and B10.A(5R) mice (designated A₂^k and A₂^b, respectively) are distinguishable by their tryptic peptide maps (Fig. 2a), as are the A_β chains isolated from B10.A(4R) and B10.A(5R) mice (designated A_β^k and A_β^b, respectively) (Fig. 3a). Peptide mapping of the A₂ and A_β chains immunoprecipitated from [B10.A(4R) × B10.A(5R)]F₁ mice with monoclonal antibody 10-2.16 directed against the I-A molecule of the B10.A(4R) parent (I-A^k) reveals that they consist solely of the A_β^k chain (Fig. 3b) and a mixture of A₂^k and A₂^b chains (Fig. 2b). The absence of an A_β^b chain in the immunoprecipitate is not due to the inability of these F₁ mice to express the A_β^b chain; antiserum directed against I-A^b antigens precipitates the A_β^b chain from F₁ mice.

We conclude from these results that the allospecificity Ia.17 recognized by monoclonal antibody 10-2.16 is determined by the β chain according to the following rationale: when a monoclonal reagent specific for I-A from parent 1 is used to precipitate I-A molecules from an F₁ offspring, several results are possible. If the specificity is determined only by the α chain, the α chain from parent 1 but not from parent 2 would be found in the F₁ precipitate. Conversely, if only the β chain determines the specificity, the β chain of parent 1 but not of parent 2 would be found. Finally, if both chains are responsible, both α and β chains from parent 1 and none from parent 2 would be found. Because the antibody displays specificity for the A_β^k chain and none for the α chains, we conclude that the β chain determines the antigenic specificity recognized by the monoclonal antibody. The observation that both α chains are found in the F₁ indicates that both α chains can freely associate with the A_β^k chain. Immunoprecipitation of both α chains cannot be due to their recognition by the monoclonal antibody, because then the monoclonal antibody would be expected to react with and precipitate I-A molecules from B10.A(5R) mice, which it does not (Table 1 and Fig. 1). However, we cannot rule out the possibility that the antigenic determinant recognized by monoclonal antibody 10-2.16 actually resides on the α chain and is generated when either A₂^k or A₂^b associate with the specific A_β^k chain.

Thus, only one of the two subunits of I-A subregion antigens, the β chain, has a crucial role in determining the antigenic determinant recognized by the monoclonal antibody. This, combined with studies that attribute the molecular polymorphism of the murine I-E subregion and K, D gene products, and their respective human analogues the HLA-DR¹⁹⁻²¹ and HLA-A,B,C^{22,23} antigens, to one of two

subunits, makes it tempting to propose that only one chain, the β chain, is generally responsible for the alloantigenic specificities of I-A antigens. To substantiate this hypothesis, reagents against several other I-A specificities must be tested.

Assignment of the alloantigenic specificities of I-A molecules to one subunit has important functional implications. Genetic²⁴ and serological²⁵ studies suggest that T-cell recognition of allotype-specific I-A molecules on B cells and macrophages is a prerequisite for the development of optimal immune responses. If T cells perceive I-A antigens with a specificity similar to antibody, it is also possible that only the β chain determines allotype-specific recognition of I-A molecules by T cells. The free association of both α components with the A_β^k component may be interpreted to mean that both α components have a common effector function and may be substituted for one another.

In contrast to the proposal that the β subunit alone determines I-A antigenic specificities, the findings of Fatham *et al.*²⁶ may be construed to suggest that some antigenic specificities perceived by T cells are due to unique combinations of α and β chains. These authors found that some cell-surface antigenic determinants controlled by the MHC and recognized by T cells in mixed lymphocyte culture (MLC) reactions, are unique to F₁ animals and absent from both parental types. Although some of these specificities may be attributed to complementation of two MHC genes required for the expression of I-E molecules, as has been demonstrated by several investigators^{27,28}, others cannot be explained on this basis. The observation that α and β chains from an F₁ animal are capable of associating in a random manner introduces the possibility of generating antigenic specificities unique to the F₁ offspring and absent from both types, and may explain the findings of Fatham *et al.* If α and β chains can reassociate in a random fashion, F₁ animals should have four distinct sets of I-A complexes, two that are unique to the F₁ and not found on either parental types and one from each parental type. From a functional viewpoint, if I-A molecules are perceived by T cells in both MLC reactions and T-B or T-macrophage cell interactions in a similar fashion, an F₁

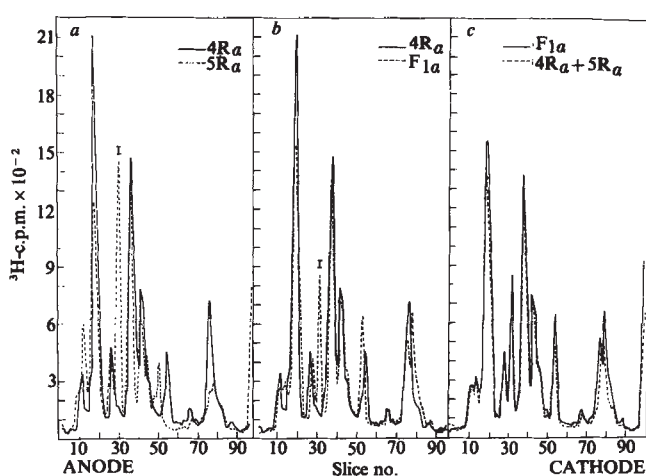


Fig. 2 Comparative tryptic peptide maps of the A₂ chains immunoprecipitated from B10.A(4R), B10.A(5R) and [B10.A(4R) × B10.A(5R)]F₁ mice. The A₂ chain isolated from B10.A(5R) mice (designated A₂^b) displays a peptide peak (I) that is absent from the A₂ chain isolated from B10.A(4R) mice (designated A₂^k) (a). The peptide map of the A₂ chain isolated from [B10.A(4R) × B10.A(5R)]F₁ mice displays peak I, which is unique to the A₂^b chain (b). Furthermore, this peptide map is virtually identical to that derived from a mixture of the A₂^k and A₂^b chains (c). Positional differences (1–2 gel slices) of peptides and the apparent 'splitting' of the peak near slice 80 (c) are due to minor variations in gel slicing.

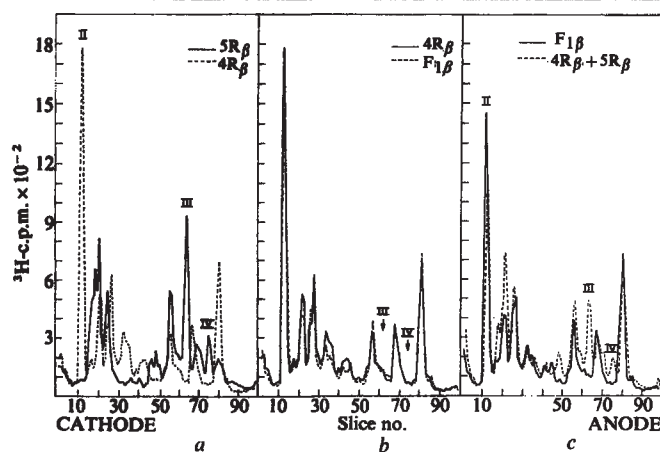


Fig. 3 Comparative tryptic peptide maps of the A_β chains immunoprecipitated from B10.A(4R), B10.A(5R) and [B10.A(4R) $\times$ B10.A(5R)] F_1 mice. The A_β chain isolated from B10.A(5R) mice (designated A_β^5) displays several unique peptides (III and IV); the A_β chain isolated from B10.A(4R) mice (designated A_β^4) also has one unique peptide (II) (a). The peptide maps of A_β chains isolated from [B10.A(4R) $\times$ B10.A(5R)] F_1 and B10.A(4R) mice are virtually identical (b). Clearly, the A_β chain isolated from [B10.A(4R) $\times$ B10.A(5R)] F_1 mice does not comprise a mixture of A_β^4 and A_β^5 chains, as its peptide map is different from that derived from the mixed A_β^4 and A_β^5 chains (c).

animal might be expected to possess some T cells that are capable of interacting with B cells and macrophages only from F_1 animals and not from either parental type. Although experiments by others clearly demonstrate that cells of F_1 animals can interact with cells of either parental type²⁹⁻³¹, functional studies designed specifically to determine the possibility of F_1 specific interactions have not been performed. It is important to discover whether such unique F_1 determinants frequently have an important role or are only found in rare cases.

The analytical method used in the present studies provides a general means of determining which chain confers the unique allospecificity on any complex consisting of multiple subunits, provided that both subunits from both parents are distinguishable and that monoclonal antibody or an operationally monospecific alloantiserum directed against the allospecificities is available. Furthermore, this method does not require retention of alloantigenicity after separation of noncovalently associated subunits to determine which subunit carries the allospecificities. Additional studies with several monoclonal antibodies directed against I-A^k and other allospecificities will enable us to assess definitively the relative contributions of the A_α and A_β polypeptides to the alloantigenicity of I-A subregion antigens.

This work was supported by NIH grant CA 23030. J.S. holds an established investigatorship from the American Heart Association. Hybridoma 10-2.16 was donated by Dr Len Herzenberg.

Received 12 December 1979; accepted 18 April 1980.

- Shreffler, D. C. & David, C. S. *Adv. Immun.* **20**, 125-195 (1975).
- Silver, J. & Hood, L. E. *Nature* **249**, 764-765 (1974).
- Silver, J. & Hood, L. E. *Nature* **256**, 63-64 (1975).
- Cullen, S. E., Freed, J. H. & Nathenson, S. G. *Transplant. Rev.* **30**, 236-270 (1976).
- Silver, J., Cocka, J. M., McMillan, M. & Hood, L. E. *Cold Spring Harb. Symp. quant. Biol.* **41**, 369-377 (1976).
- Silver, J., Russell, W. A., Reis, B. L. & Frelinger, J. A. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5131-5134 (1977).
- McMillan, M., Cocka, J. M., Murphy, D. B., McDevitt, H. O. & Hood, L. E. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5135 (1977).
- Cook, R. G., Uhr, J. W., Capra, J. D. & Vitetta, E. S. *J. Immun.* **121**, 2205-2212 (1978).
- Silver, J. & Hood, L. E. *Proc. natn. Acad. Sci. U.S.A.* **73**, 599-603 (1976).
- Silver, J., Walker, L. E., Reisfeld, R. A., Pellegrino, M. A. & Ferrone, S. *Molec. Immun.* **16**, 37-42 (1979).
- McMillan, M., Cocka, J. M., Murphy, D. B., McDevitt, H. O. & Hood, L. E. *Immunogenetics* **6**, 137-147 (1978).

- Freed, J. H. & David, C. S. in *Current Trends in Histocompatibility* (eds Reisfeld, R. A. & Ferrone, S.) (Plenum, New York, in the press).
- Cook, R. G., Vitetta, E. S., Uhr, J. W. & Capra, J. D. *Molec. Immun.* **16**, 29-35 (1979).
- Silver, J. & Russell, W. A. *Immunogenetics* **8**, 339-346 (1979).
- Cook, R. G., Capra, J. D., Bednarczyk, J. L., Uhr, J. W. & Vitetta, E. S. *J. Immun.* **123**, 2799-2803 (1979).
- Silver, J. in *Current Trends in Histocompatibility* (eds Reisfeld, R. A. & Ferrone, S.) (Plenum, New York, in the press).
- Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A. & Herzenberg, L. A. *Curr. Topics Microbiol. Immun.* **81**, 115-129 (1978).
- Silver, J. *Transplant. Proc.* **9**, 661-665 (1977).
- Silver, J. & Ferrone, S. *Nature* **279**, 436-437 (1979).
- Silver, J. & Russell, W. A. *Nature* **279**, 437-438 (1979).
- Silver, J. & Ferrone, S. *Immunogenetics* **10**, 295-298 (1980).
- Grey, H. M. *et al. J. exp. Med.* **131**, 1608-1612 (1973).
- Nakamuro, K., Tanigaki, M. & Pressman, D. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2863-2865 (1973).
- Katz, D. H., Graves, M., Dorf, M. E., Dimuzio, H. & Benacerraf, B. *J. exp. Med.* **141**, 263-268 (1975).
- Frelinger, J. A., Niederhuber, J. E. & Shreffler, D. C. *Science* **188**, 268-270 (1975).
- Fathman, C. G., Watanabe, T. & Augustin, A. *J. Immun.* **121**, 259-264 (1978).
- Jones, P. P., Murphy, D. B. & McDevitt, H. O. *J. exp. Med.* **148**, 925-939 (1978).
- Silver, J. *J. Immun.* **123**, 1423-1425 (1979).
- Paul, W. E., Shevach, E. M., Pickeral, S., Thomas, D. W. & Rosenthal, A. S. *J. exp. Med.* **145**, 618-630 (1977).
- Swierkosz, J. E., Rock, K., Marrack, P. & Kappler, J. W. *J. exp. Med.* **147**, 554-570 (1978).
- Sprent, J. *J. exp. Med.* **147**, 1142-1158 (1978).
- Feeney, A. J. & Hämmerling, U. *Immunogenetics* **3**, 369-379 (1976).

Increased proton conductance pathway in brown adipose tissue mitochondria of rats exhibiting diet-induced thermogenesis

Sarah L. Brooks*, Nancy J. Rothwell*, Michael J. Stock*, Anne E. Goodbody† & Paul Trayhurn†

*Department of Physiology, St. George's Hospital Medical School, London SW17 0RE, UK

†Dunn Nutrition Laboratory, University of Cambridge and MRC, Cambridge CB4 1XJ, UK

It has recently been demonstrated¹ that in rats induced to overeat by being fed a varied and palatable diet (the 'cafeteria diet') there is a marked increase in heat production which serves to reduce, or prevent, the development of obesity. This diet-induced thermogenesis is associated with increases in sympathetic activity, and with changes in brown adipose tissue. Following cafeteria feeding, brown adipose tissue hypertrophies and exhibits increased lipolysis and an apparently greater thermogenesis in response to noradrenaline. These metabolic changes resemble those seen during non-shivering thermogenesis in cold-adapted rats, and it was proposed¹ that non-shivering thermogenesis and diet-induced thermogenesis have a similar metabolic origin which depends on the unique capacity of brown adipose tissue for thermogenesis. During non-shivering thermogenesis heat is produced in brown adipose tissue through a proton conductance pathway across the inner mitochondrial membrane that dissipates the proton gradient generated by respiration². The activity of the proton conductance pathway can be modulated by purine nucleotides, and changes in the pathway seem to be related to the level of purine nucleotide binding to brown adipose tissue mitochondria. We now report results which indicate that the proton conductance pathway is augmented in cafeteria-fed rats, and suggest that it operates to dissipate their excess energy intake through diet-induced thermogenesis.

In the first experiments, the activity of mitochondrial enzymes and the effect of purine nucleotides on mitochondrial respiration were determined with brown adipose tissue of normal and cafeteria-fed animals. Groups of adult, male Sprague-Dawley rats (Charles River) were maintained on either the conventional stock diet (PRD, Christopher Hill Group) or the cafeteria diet (see refs 1, 3 for details) for up to

20 days in a room maintained at 24°C. As in previous studies¹, the cafeteria-fed rats overate and exhibited increased metabolic rates and an increased thermogenic response to injected doses of noradrenaline. After 10–20 days the rats were killed and the interscapular brown adipose tissue was removed and homogenized, and the total protein content and cytochrome oxidase (EC 1.9.3.1.) activity were measured. Mitochondria prepared from homogenates were also assayed for protein, and for cytochrome oxidase and α -glycerophosphate dehydrogenase (EC 1.1.1.99.5.) activity. Details of the methods used and the results obtained are shown in Table 1.

Cafeteria feeding resulted in a near doubling of interscapular brown adipose tissue (BAT) mass and total protein content. There were also large increases in the total activity of the two mitochondrial respiratory enzymes, related to a twofold increase in mitochondrial protein content of the brown adipose tissue of cafeteria-fed rats. The enzyme changes were not due to increases in specific activity (that is, activity per mg mitochondrial protein) nor to differences in the recovery of mitochondria, and indicate that the oxidative capacity of this brown adipose tissue depot increases substantially during overfeeding.

These changes resemble those seen in cold-adapted animals^{4–6} where high rates of mitochondrial respiration in brown adipose tissue result from a combination of increased oxidative capacity and reduced respiratory control². Respiratory control can be partially restored by incubating mitochondria in the presence of albumin but significant improvements require the further addition of purine nucleotides⁷. In the present study, respiratory rates of brown adipose tissue mitochondria from cafeteria-fed rats were 30% greater than those from control animals, even when incubated with albumin to avoid differences due to any uncoupling effect of endogenous fatty acids (Table 2). However, the addition of

Table 2 Respiration of mitochondria from brown adipose tissue of control and cafeteria-fed rats

	Mitochondrial respiration (nmol O ₂ per mg protein per min)	
	Control	Cafeteria
+ Albumin (5 mg ml ⁻¹)	32.8 ± 2.0	42.7 ± 2.6*
+ GDP (1 mM)	20.2 ± 1.2	18.7 ± 2.3 NS
+ FCCP (5 µM)	35.8 ± 2.6	38.3 ± 3.4 NS

The age and weight of the rats used were similar to those in Table 1. Mitochondria were prepared by the method of Slinde *et al.*¹⁶, and oxygen consumption was measured at 25°C in a Clark-type oxygen electrode (Rank Brothers) using the conditions described by Nicholls⁷. Mitochondrial protein was measured as in Table 1. Results are mean values ± s.e.m., *n* = 8. NS, Not significantly different (*P* > 0.05) from controls.

**P* < 0.01.

GDP (Sigma) resulted in a marked 'tightening' of respiratory control such that respiratory rates were decreased in both groups and the differences between them were abolished. This effect of GDP could be overcome by adding the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, Sigma). These results indicate that brown adipose tissue mitochondria from cafeteria rats are more 'loosely coupled' than those from control rats.

The action of purine nucleotides on mitochondrial respiratory control in BAT is due to inhibition of the proton conductance pathway². This inhibition is associated with the binding of the purine nucleotides to an inner membrane protein (molecular weight 32,000) unique to brown adipose tissue mitochondria. Changes in mitochondrial GDP binding have been used to study the capacity of the proton conductance pathway during cold-adaptation in rats^{8,9}, and during early development in both rats⁹ and guinea pigs¹⁰, as well as to investigate abnormalities in heat production in brown adipose tissue from genetically obese (*ob/ob*) mice¹¹. In view of the similarities between non-shivering thermogenesis and diet-induced thermogenesis an experiment was carried out to compare the purine nucleotide binding capacity of brown adipose tissue mitochondria isolated from cafeteria- and stock-fed rats maintained in warm and cold environments.

Similar rats to those used in the first experiments were housed at either 24°C (warm-adapted) or 4°C (cold-adapted) and fed the stock diet for 15 days, after which time half the animals at each environmental temperature were allowed access to the cafeteria diet. We have previously shown¹² that

Table 1 Mitochondrial enzyme activities in interscapular brown adipose tissue from control and cafeteria-fed rats

	Control	Cafeteria
Weight of animals (g)	196.5 ± 3.3	190.7 ± 2.5 NS
Interscapular brown adipose tissue mass (mg)	275 ± 10	538 ± 18*
Total brown adipose tissue protein (mg)	32.4 ± 2.4	58.7 ± 4.0*
Total cytochrome oxidase activity (µmol cytochrome <i>c</i> oxidized per min per mg tissue)	19.8 ± 3.3	44.2 ± 7.1†
Mitochondrial cytochrome oxidase activity (µmol cytochrome <i>c</i> oxidized per min per total mitochondrial preparation)	5.1 ± 0.7	11.8 ± 1.5†
α -Glycerophosphate dehydrogenase activity (µmol O ₂ consumed per min per total mitochondrial preparation)	0.58 ± 0.03	1.62 ± 0.19*

The animals used were aged approximately 6 weeks. Protein content of tissue homogenates and mitochondrial preparations was determined using a protein dye reagent kit with a bovine serum albumin standard (Bio-Rad). Mitochondria were prepared using the isolation procedure described by Slinde *et al.*¹⁶. Cytochrome oxidase activity was determined spectrophotometrically using a modification of the assay described by Yonetani and Ray¹⁷ and α -glycerophosphate dehydrogenase activity was assayed polarographically¹⁸. The specific activities of the two enzymes (that is, activity per mg mitochondrial protein) were not different in the two groups but the amount of mitochondrial protein isolated was different—3.5 ± 0.3 and 7.1 ± 0.4 mg in control and cafeteria-fed groups, respectively (*P* < 0.001). Comparison of cytochrome oxidase activities in whole tissue homogenates and mitochondrial preparations indicates that the fractional yield of mitochondria was identical in both groups. Results are mean values ± s.e.m., *n* = 8. NS, Not significant (*P* > 0.05).

**P* < 0.001; †*P* < 0.01, compared with control values.

Table 3 Purine nucleotide (GDP) binding to interscapular brown adipose tissue mitochondria from cafeteria and control rats maintained at either 24°C (warm) or 4°C (cold)

	Warm control	Warm cafeteria	Cold control	Cold cafeteria
Animal weights (g)	326.3 ± 18.1	306.0 ± 25.5	296.6 ± 11.8	327.4 ± 21.2
Mitochondrial protein recovered (mg)	5.7 ± 0.9	8.7 ± 0.9*	17.2 ± 1.4‡	21.0 ± 1.3‡
GDP binding (pmol GDP per mg mitochondrial protein)	52.6 ± 7.4	135.3 ± 24.0†	249.8 ± 17.8‡	237.5 ± 14.6‡
<i>n</i>	7	5	5	5

The rats used in these experiments were aged approximately 10 weeks. Mitochondria were prepared by the method of Slinde *et al.*¹⁶, and the protein content of each preparation was determined colorimetrically by a modified Lowry assay¹⁹ using bovine serum albumin as a standard. Mitochondrial GDP-binding was measured essentially as described by Nicholls²⁰ with the modifications of Desautels *et al.*⁶; ³H-GDP and ¹⁴C-sucrose (both Radiochemical Centre) incubations were carried out for 7 min at room temperature. Results are mean values ± s.e.m.

**P* < 0.05; †*P* < 0.01; ‡*P* < 0.001, compared with warm controls.

cafeteria feeding in the warm produces quantitatively similar increases in metabolic rate, noradrenaline sensitivity and brown adipose tissue mass, to those seen with cold-adapted stock-fed controls. In the present study, animals were killed after 10–20 days on the experimental diets and brown adipose tissue mitochondria prepared for determination of GDP-binding capacity. Details of the methods used and results obtained are shown in Table 3.

Cafeteria feeding in the warm environment resulted in a significant 2.5-fold increase in GDP-binding per mg mitochondrial protein compared with warm-adapted controls. Thus, the high diet-induced thermogenesis resulting from cafeteria feeding apparently leads to a marked increase in the proton conductance of brown adipose tissue mitochondria, similar to that observed with cold-adaptation. It is unlikely that the increased GDP binding of cafeteria-fed rats over that of the controls was due to the isolation of a different population of mitochondria or to differences in the purity of the preparations, because the specific activities of the two respiratory enzymes (cytochrome oxidase and α -glycerophosphate dehydrogenase) were almost identical for both groups.

Cold-adaptation produced a greater than fourfold increase

in GDP binding per mg mitochondrial protein in both stock- and cafeteria-fed rats. The results for the cold-adapted control group are similar to those reported by Desautels *et al.*⁸, and although an additive effect of cold and cafeteria feeding on metabolic rate and noradrenaline sensitivity has been seen in earlier experiments¹², this is not reflected in the GDP-binding results for the cold-adapted cafeteria group in the present study.

The present results are the first report of an effect of diet and energy intake on the capacity of brown adipose tissue mitochondria for thermogenesis. They support our earlier suggestion¹ that the mechanisms of diet-induced thermogenesis and non-shivering thermogenesis are similar, and are consistent with the improved cold tolerance of overfed rats¹².

Genetically obese animals (*ob/ob* and *db/db*) have impaired thermogenesis^{13–15}, which has been associated with a lowered purine nucleotide binding in their brown adipose tissue mitochondria¹¹. This finding, together with the results of the present study, suggest that brown adipose tissue has an important regulatory role in energy balance, as well as in body temperature.

This work was supported by the MRC, the Rank Prize Funds and SW Thames Regional Health Authority.

Received 21 February; accepted 2 May 1980.

1. Rothwell, N. J. & Stock, M. J. *Nature* **281**, 31–35 (1979).
2. Nicholls, D. G. *Biochim. biophys. Acta* **549**, 1–29 (1979).
3. Rothwell, N. J. & Stock, M. J. *J. comp. Physiol. Psychol.* **93**, 1024–1034 (1979).
4. Jansky, L., Votapková, Z. & Feiglva, E. *Physiologia bohemoslov.* **18**, 443–451 (1969).
5. Barnard, J., Skala, J. & Lindberg, O. *Comp. Biochem. Physiol.* **33**, 499–508 (1970).
6. Heick, H. M. C., Vachon, C., Kallai, M. A., Begin-Heick, N. & Leblanc, J. *Can. J. Physiol. Pharmacol.* **51**, 751–758 (1973).
7. Nicholls, D. G. *Eur. J. Biochem.* **49**, 573–583 (1974).
8. Desautels, M., Zaror-Behrens, G. & Himms-Hagen, J. *Can. J. Biochem.* **56**, 378–383 (1978).
9. Sundin, U. & Cannon, B. *Comp. Biochem. Physiol.* **65B**, 463–471 (1980).
10. Rafael, J. & Heldt, H. W. *FEBS Lett.* **63**, 304–308 (1976).
11. Himms-Hagen, J. & Desautels, M. *Biochem. biophys. Res. Commun.* **83**, 628–634 (1978).
12. Rothwell, N. J., Schönbaum, E., Smith, I. C. H. & Stock, M. J. *J. Physiol., Lond.* **300**, 458 (1980).
13. Trayhurn, P., Thurlby, P. L. & James, W. P. T. *Nature* **266**, 60–62 (1977).
14. Trayhurn, P. & James, W. P. T. *Pflügers Arch. ges. Physiol.* **373**, 189–193 (1978).
15. Trayhurn, P. *Pflügers Arch. ges. Physiol.* **380**, 227–232 (1979).
16. Slinde, E., Pedersen, J. I. & Flatmark, T. *Analyt. Biochem.* **65**, 581–585 (1975).
17. Yonetani, T. & Ray, G. S. *J. biol. Chem.* **240**, 3392–3398 (1965).
18. Gurr, M. I., Mawson, R., Rothwell, N. J. & Stock, M. J. *J. Nutr.* **110**, 532–542 (1980).
19. Schacterle, G. R. & Pollack, R. L. *Analyt. Biochem.* **51**, 654–655 (1973).
20. Nicholls, D. G. *Eur. J. Biochem.* **62**, 223–228 (1976).

Insulin stimulates sugar transport in giant muscle fibres of the barnacle

P. F. Baker & A. Carruthers

Department of Physiology, University of London King's College, Strand, London WC2R 2LS, UK

Insulin stimulates sugar transport in vertebrate skeletal muscle but the mechanism of insulin action is unknown¹. It has been reported that Na transport in giant muscle fibres of the barnacle (*Balanus nubilis*) is sensitive to insulin² but no one has examined the sensitivity of sugar transport to insulin in this preparation. We show here that insulin does, indeed, stimulate sugar transport in barnacle muscle. The great advantage of barnacle muscle over all other muscles used so far for investigating the mechanism of insulin action is its large size, which facilitates measurements on single cells and permits the experimenter to control the intracellular environment of the muscle fibre by the technique of internal dialysis³. Using single muscle fibres it is possible to show that acceleration of sugar transport by insulin is associated with a fall in ionized Ca, a fall in cyclic AMP and a rise in cyclic GMP. Working with internally dialysed muscle fibres we find that insulin only increases sugar transport when the dialysis solution contains ATP. In the absence of insulin, sugar transport in dialysed muscle is increased by a rise in ionized Ca, a fall in cyclic AMP and, when the internal Ca is elevated, by a rise in cyclic GMP.

D-Glucose is taken up and metabolised by barnacle muscle fibres. For this reason, the non-metabolised sugar 3-O-methylglucose was used throughout the present study. In addition to their sensitivity to insulin, 3-O-methylglucose fluxes in barnacle muscle show several features in common with sugar transport in mammalian tissues. Sugar uptake and

exit consist of two components: a saturable component (apparent $K_m = 5$ mM, $V_{max} = 17$ pmol cm⁻² s⁻¹) which is reduced by both phloretin (apparent $K_i = 10^{-4}$ M) and cytochalasin B (apparent $K_i = 5 \times 10^{-6}$ M), and a component which increases linearly with sugar concentration and is insensitive to phloretin or cytochalasin B.

Porcine insulin stimulates reversibly the rate of sugar uptake and exit in isolated muscle fibres in a concentration-dependent manner (half-maximal stimulation seen at 1.2 U ml⁻¹). Barnacle muscle fibres bind ¹²⁵I-insulin: this can be displaced by cold insulin, and in the presence of 1 U ml⁻¹, the insulin space of single fibres is about five times greater than that of mannitol or sucrose. Both insulin binding and the activation of sugar transport become saturated at roughly the same concentration of insulin and on the assumption that all the radioactivity is bound to the surface of the muscle, the number of insulin binding sites is 600 per μ m². These findings suggest that barnacle muscle fibres have receptors for insulin but we do not know whether barnacles possess endogenous insulin or an insulin-like peptide. The stimulatory action of insulin is confined to the phloretin- or cytochalasin B-sensitive component of sugar transport. In the presence of these inhibitors, insulin has no effect on sugar fluxes but in their absence it increases the rate of sugar uptake 2.4-fold and the rate of sugar exit approximately 2.5-fold. Denatured insulin, ZnCl₂ (60 μ M) and albumin (0.5 mg ml⁻¹) are unable to mimic the effect of native insulin. Analysis of the kinetics of sugar uptake shows that insulin increases the V_{max} with little or no effect on the apparent K_m .

Insulin has been claimed to exert its action on sugar transport through changes in cyclic nucleotide levels or ionized Ca^{1,2,4–6}. These possibilities can be examined directly using single barnacle muscle fibres. The effect of insulin on cyclic nucleotide levels is shown in Fig. 1. Insulin (5 U ml⁻¹) elicits a roughly simultaneous rise in cyclic GMP and fall in cyclic AMP levels which can be detected as early as 2 min after

Fig. 1 *a-d* The effects of insulin (5 U ml^{-1}) on 3-*O*-methylglucose (3-*O*-MG) exit, cyclic nucleotide levels and aequorin light output in barnacle muscle. *a*, Time course of the stimulation of sugar exit in single muscle fibres by insulin. Fibres were bathed in seawater, composition (mM): NaCl, 442; KCl, 10; MgCl_2 , 53; CaCl_2 , 11; Tris, 10, pH 7.6. Each point consists of four or more fibres. Mean fibre diameter, $1,326 \mu\text{m}$; temperature, $11 \pm 1^\circ\text{C}$. *b*, Time course of the rise in cyclic GMP levels caused by insulin. Each point consists of six separate determinations. The concentration of cyclic GMP following 20 min incubation in insulin-free seawater ($\bullet$) is shown below the points. Temperature, 12°C . Recovery of exogenous cyclic GMP added to barnacle muscle homogenate was 89.2%. *c*, Time course of the fall in cyclic AMP levels caused by insulin. Each point consists of five or six separate determinations. Cyclic AMP levels corresponding to 20 min incubation in seawater ($\square$) are shown above the points. Temperature, 12°C . Recovery of exogenous cyclic AMP added to barnacle muscle homogenates was 93.4%. *d*, Time course of the change in intracellular free Ca as a result of exposure to insulin. The fibre was injected with aequorin and left in flowing seawater for 2 h to allow the aequorin to diffuse uniformly across the cell. The figure is a tracing of the photomultiplier output as displayed on a pen recorder. The light output was stable before application of insulin. Above the record at 20 min is shown the level to which light output recovered after 20 min in insulin-free seawater. Fibre diameter, $984 \mu\text{m}$; temperature, 11°C .

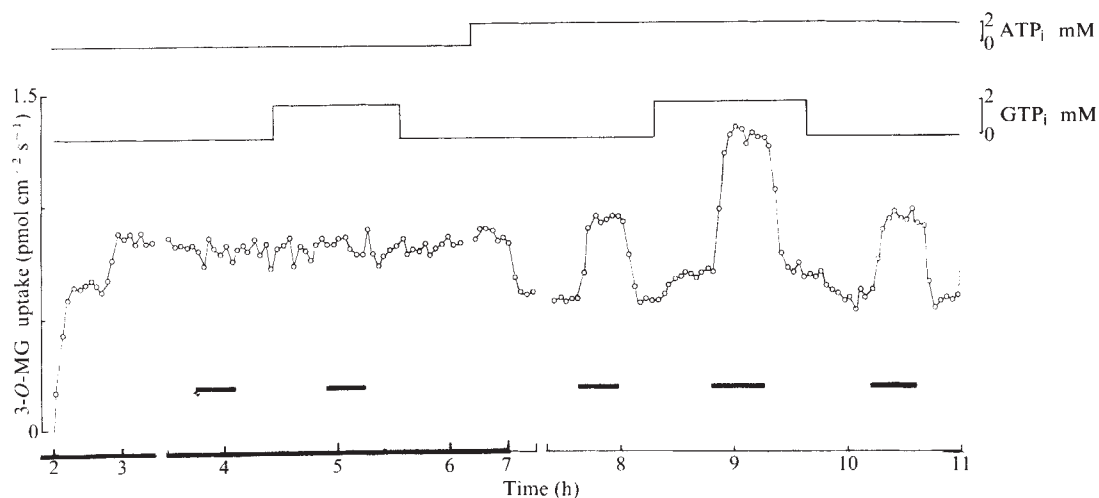
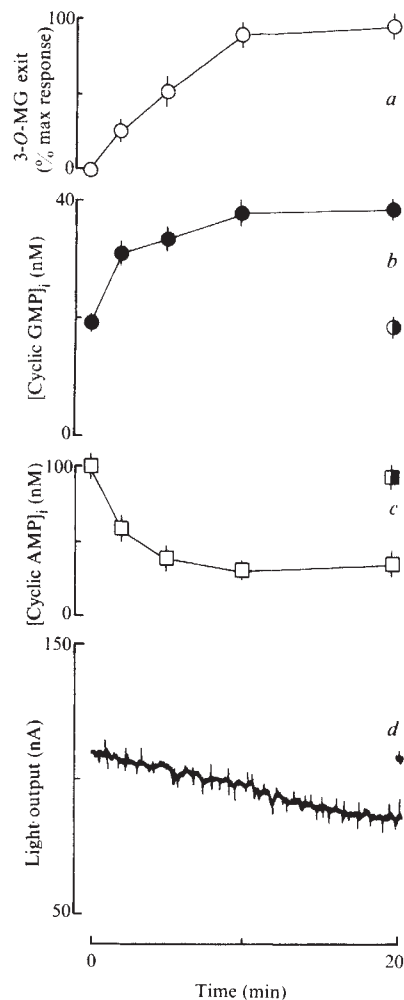


Fig. 2 The effect of insulin on 3-*O*-methylglucose uptake in an internally dialysed muscle fibre. The fibre was dialysed with a solution of the following composition (mM): K aspartate, 150; NaCl, 32; MgCl_2 (in excess of ATP), 10; CaCl_2 , 1.5; EGTA, 5; HEPES, 5; PIPES, 5; NaCN, 2; ECCP, $2 \mu\text{g ml}^{-1}$; phenol red, 2, pH 7.0. The osmolarity was adjusted to 975 mosM with sucrose. ATP was added to the solution as MgATP. In calculating the concentration of ionized Ca, the log of the stability constant for Ca-EGTA at pH 7.0 was taken as -6.723 . Internal ATP and GTP concentrations are shown above the points. External application of insulin (5 U ml^{-1}) is indicated by the solid bars. The external 3-*O*-methylglucose concentration was 2 mM in all dialysis experiments. The fibre was dialysed for 2 h with a solution containing 0 ATP before external application of isotope. Fibre diameter, $985 \mu\text{m}$; temperature, 10°C . As in Fig. 4, sugar uptake is expressed as $\text{pmol cm}^{-2} \text{ s}^{-1}$.

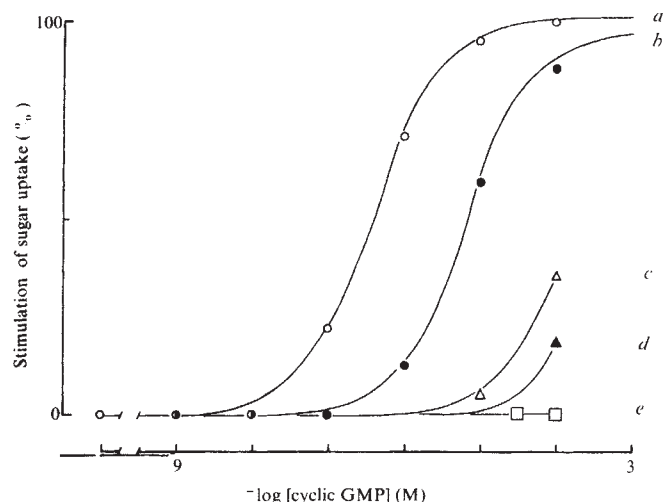


Fig. 3 The effect of raised internal free Ca on the activation of 3-O-methylglucose uptake by cyclic GMP. The data are pooled from six fibres. Each curve shows the cyclic GMP-dependent activation of sugar uptake at different Ca levels. The small activation caused by raising the internal ionized Ca concentration is subtracted from each point. The ionized Ca levels are 0.91, 0.5, 0.41, 0.27 and $0.12 \mu\text{M}$ for curves a–e, respectively. Curves a–c are calculated by Lineweaver–Burk analysis, on the assumption that the maximum stimulation in all cases is 100%. The apparent Michaelis constants for cyclic GMP are: curve a, $4.12 \times 10^{-7} \text{ M}$; curve b, $6.92 \times 10^{-6} \text{ M}$; curve c, $1.9 \times 10^{-4} \text{ M}$. Curves d and e are drawn by eye. Mean fibre diameter $1,241 \mu\text{m}$; temperature, 10°C .

adding insulin to the medium. The changes in cyclic nucleotide levels seem to precede the change in the rate of sugar exit; this is not inconsistent with the possibility that insulin increases sarcolemmal sugar permeability through alterations in cyclic nucleotide levels.

The hypothesis that insulin acts in muscle by mobilizing intracellular Ca can be tested directly in the barnacle muscle fibre using the Ca-sensitive photoprotein aequorin. Fibres were injected axially with aequorin and the light emitted passed along a fibre optic system to a photomultiplier (EMI, 9635 B) the output of which was displayed on a pen recorder. Resting light output was calibrated by use of a variety of Ca-EGTA buffers⁷ and, on the assumption of an internal ionized

Mg of 4 mM (ref. 8) and pH of 7.2 (ref. 9), the light output was found to correspond to a free internal Ca of 70 nM . On exposing fibres loaded with aequorin and isotopic 3-O-methylglucose to insulin (5 U ml^{-1}), there was a simultaneous increase in the rate of sugar exit and a small, but reproducible, fall in the resting light output (Fig. 1). This effect on light output was not mimicked by ZnCl_2 ($60 \mu\text{M}$), denatured insulin or albumin (0.5 mg ml^{-1}). A fall in light output was rather unexpected in view of the claims that insulin may increase intracellular ionized Ca. It is well known that intracellular ionized Ca can be manipulated by altering external Ca or replacing external Na with Li, and the effects of these manoeuvres on sugar fluxes were examined. Removal of external Ca reduced light output by some 70% and replacement of Na by Li increased light output eight-fold; however, sugar exit in both conditions was unaffected. We conclude that although insulin seems to reduce internal free Ca in barnacle muscle, its effect on sugar transport in this preparation seems not to be related in any simple way to the myoplasmic ionized Ca concentration.

Another way to examine this problem is to use internally dialysed muscle fibres³. With this technique it is possible to clamp the ionized Ca to some predetermined level using Ca-EGTA buffers; if a change in ionized Ca is a prerequisite of insulin action, it should be impossible to elicit any response to insulin in conditions of dialysis with solutions containing EGTA buffers. This was certainly not the case. Figure 2 shows that, provided ATP was in the dialysis solution, insulin stimulated the rate of sugar uptake in the presence of a buffered Ca concentration of 120 nM and the stimulatory effect of insulin was enhanced when GTP (2 mM) was also included in the internal solution. This action of insulin was not seen in dialysed fibres exposed to cytochalasin B. These observations suggest that although intracellular ATP is essential for insulin action, alterations in internal ionized Ca are probably not necessary.

Figure 2 also shows that ATP *per se* has an effect on sugar fluxes. In the nominal absence of ATP the uptake of sugar increased. This is consistent with the well known effects of metabolism and anoxia on sugar fluxes in muscle cells⁶. The concentration of ATP which produced half-maximal inhibition of 3-O-methylglucose uptake was 0.4 mM .

Using the dialysed muscle fibre it is possible to examine whether any of the alterations in cyclic nucleotide levels or ionized Ca brought about by application of insulin are capable

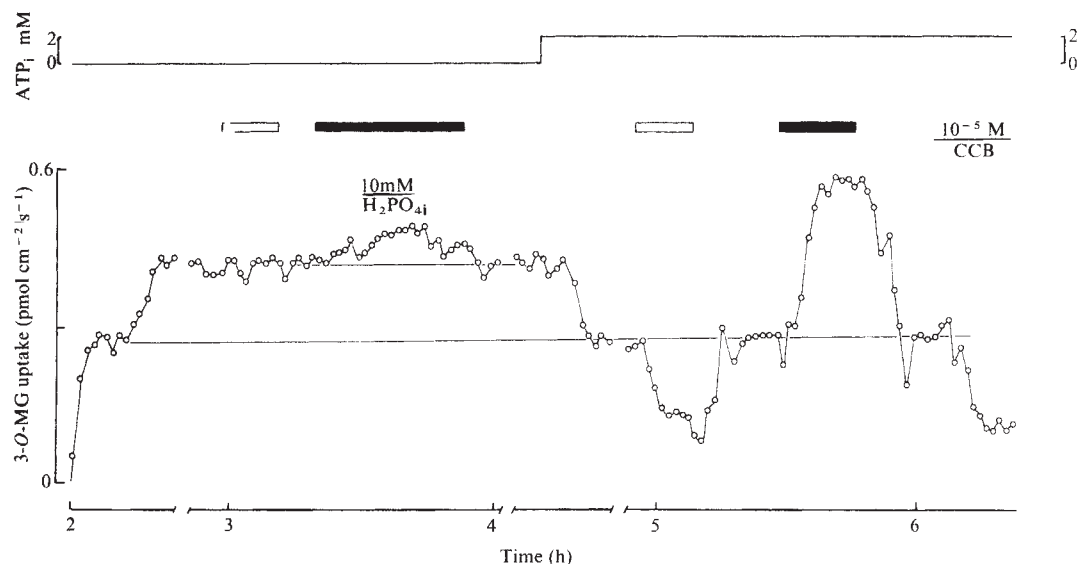


Fig. 4 Effect of cyclic nucleotides on sugar uptake in dialysed muscle. Internal solution as in Fig. 2. Internal ionized Ca = $0.91 \mu\text{M}$. The internal ATP concentrations are shown above the points. Internal application of cyclic AMP (10^{-6} M) and cyclic GMP (10^{-4} M) are indicated by the open and closed bars, respectively. Cytochalasin B (CCB) was applied externally at the end of the experiment. Fibre diameter, $1,098 \mu\text{m}$; temperature 10°C .

themselves of mimicking the action of insulin on sugar fluxes. Raising internal Ca from 0.12 to 0.91 μM produced a small increase in the rate of sugar uptake averaging 1.27-fold. It follows that a fall in ionized Ca as seen after application of insulin should, if anything, reduce sugar uptake.

Internal application of cyclic AMP reduced the rate of sugar uptake in dialysed muscle. When cyclic AMP (100 μM) was added to an internal solution containing 2 mM ATP and a free Ca of 0.12 μM , 3-O-methylglucose uptake was reduced to $29.1 \pm 4.5\%$ of control values ($n=5$). This inhibition of uptake was half maximal at 10^{-6} M cyclic AMP_i and was independent of the internal free Ca concentration.

When the internal free Ca was low (0.12 μM), internal application of cyclic GMP had no effect on the rate of sugar uptake. On raising the internal ionized Ca from 0.12 to 0.91 μM , however, cyclic GMP_i (100 μM) rapidly and reversibly stimulated the rate of 3-O-methylglucose uptake 2.13-fold ($n=4$). Raising internal free Ca seemed to increase the affinity of the system for cyclic GMP. Thus, increasing internal free Ca from 0.5 to 0.91 μM reduced the concentration at which cyclic GMP stimulated the rate of sugar uptake from

6.9×10^{-6} to 4.2×10^{-7} M (see Fig. 3). Figure 4 shows that ATP_i must be present for both cyclic AMP and cyclic GMP to influence the rate of sugar transport.

These results show that sugar uptake can be affected by changes in ionized Ca, cyclic AMP and cyclic GMP; however, can the measured changes in these parameters in intact muscles account quantitatively for the observed stimulation of sugar fluxes in dialysed muscles? Although the changes are somewhat smaller than those needed to alter sugar fluxes in dialysed fibres, it is unlikely that cyclic nucleotide levels are uniform across the fibre. Bearing in mind the peripheral location of adenylate cyclase in the plasma membrane, it is very possible that cyclic AMP is at appreciably higher concentrations in the myoplasm close to the sarcolemma. If this is the case, a fall in cyclic AMP could certainly play a part in mediating the increase in sugar transport brought about by insulin. The experiments on dialysed fibres suggest that alterations in cyclic GMP could be important when the background level of ionized Ca is raised, for instance during exercise; however, it is possible that dialysis removes some small molecular weight factor that is synergistic with cyclic GMP.

Received 12 January; accepted 15 April 1980.

1. Czech, M. P. A. *Rev. Biochem.* **46**, 359–384 (1977).
2. Bittar, E. E., Schultz, R. & Harkness, C. J. *Membrane Biol.* **34**, 203–227 (1977).
3. Brinley, F. J. Jr & Mullins, L. J. *J. gen. Physiol.* **50**, 2303–2331 (1967).
4. Fain, J. N. in *MTP Int. Rev. Sci., Biochem. Ser. 1* Vol. 8 (ed. Rickenberg, H. V.) 1–23 (University Park Press, Baltimore, 1974).

5. Illiano, G., Tell, G. P. E., Siegel, M. I. & Cuatrecasas, P. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2443–2447 (1973).
6. Clausen, T. *FEBS Symp.* **42**, 481–499 (1977).
7. Baker, P. F., Hodgkin, A. L. & Ridgway, E. B. *J. Physiol., Lond.* **218**, 709–755 (1971).
8. Brinley, F. J. Jr, Scarpa, A. & Tiffert, T. *J. Physiol., Lond.* **266**, 545–565 (1977).
9. Boron, W. F. & Roos, A. *Am. J. Physiol.* **231**, 799–809 (1976).

Evidence for two insulin receptor populations on human erythrocytes

Victoria Herzberg, J. Mark Boughter,
Sandra Carlisle & Donald E. Hill

Department of Pediatrics, University of Arkansas for Medical
Sciences, Little Rock, Arkansas 72201

Human erythrocytes specifically bind ^{125}I -insulin in a manner similar to cells in which insulin exerts a physiological response¹. In addition, erythrocytes are of practical value for correlating *in vitro* insulin binding with *in vivo* carbohydrate intolerance^{2,3}. The competitive binding of labelled and unlabelled insulin to erythrocyte receptors is typically curvilinear when plotted according to Scatchard⁴. The curvilinear nature of the Scatchard plot describing insulin binding to membrane receptors, although originally attributed to heterogeneous sites⁵, has been more recently interpreted as negative cooperativity between homogeneous sites⁶. Evidence reported here, however, suggests that there are two populations of insulin receptors on erythrocytes. Specific concentrations of concanavalin A (Con A), a lectin which mimics insulin activity⁷, are shown here to inhibit one population of receptors leaving another population unaffected.

As Fig. 1 shows, Con A competes with labelled insulin for the erythrocyte receptors. After increasing the concentration of Con A to 5 $\mu\text{g ml}^{-1}$, a 50% reduction in ^{125}I -insulin binding was observed. Concentrations of Con A greater than 20 $\mu\text{g ml}^{-1}$ presented problems with cell agglutination.

In Fig. 2a, the individual curvilinear Scatchard plots obtained by the competitive binding of labelled and unlabelled insulin to erythrocyte receptors are illustrated with the 'best fit' line derived by 'Ligand' (ref. 8), a computer program designed to consider simultaneous analysis of n ligands binding to m classes of binding sites. In this instance, a single ligand (insulin) was investigated. The broken lines represent two heterogeneous, non-interacting binding sites mathematically derived from the 'best fit' line, illustrating each binding species as though the other were not present. The slope of each line represents the affinity (K_1 , K_2) while the abscissa intercepts

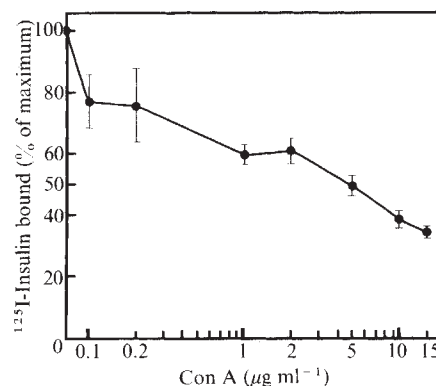


Fig. 1 Effect of Con A on the specific binding of ^{125}I -insulin to human erythrocytes. Data from five normal adult volunteers are expressed as mean $\pm$ 2s.d. Blood was collected in EDTA, and erythrocytes were purified on Isolymp (Gallard-Schlesinger) gradients¹⁰, yielding a relatively homogeneous population of mature erythrocytes (0.01% granulocytes; $\approx 1\%$ reticulocytes). Crystalline porcine insulin (Lilly) was labelled with ^{125}I to a specific activity of 125–150 $\mu\text{Ci per } \mu\text{g}$ (refs 16, 17). 1.5×10^9 erythrocytes were added to a series of tubes in which ^{125}I -insulin (0.2 ng ml^{-1}) and increasing amounts of Con A (Sigma, grade IV) were mixed with Tris-HEPES buffer (pH 8.0, 284 mosmol per kg) to give a final volume of 0.5 ml. The erythrocyte receptor assay was as described before⁴, with the following modifications: (1) ^{125}I -insulin was repurified on Sephadex G-50 immediately before use; (2) cells were incubated for 4 h (15°C) to attain equilibrium binding, and (3) non-specific binding was determined in the presence of unlabelled insulin ($10 \mu\text{g ml}^{-1}$) and was subtracted from all data points before analysis.

(R_1 , R_2) approximate the maximum binding capacity of the two receptor populations. One receptor population (I) has a high affinity and low capacity for insulin, whereas the other (II) has a low affinity and high capacity for insulin. These lines may also be derived graphically as described by Rosenthal⁹.

The experimentally determined insulin binding data obtained in the presence of Con A (Fig. 2b) approximate the mathematically derived site II. Variations in the concentration of Con A which proved optimal for linearization of the Scatchard plot were noted when erythrocytes from different

individuals were tested; however, in all cases, a linear component whose correlation coefficient varied from -0.90 to -0.98 was observed. Insulin binding in the presence of Con A therefore demonstrates complete inhibition of binding site I and clearly delineates site II. The suppression of insulin binding at site I by Con A may result from steric hindrance by the larger Con A molecule binding to a contiguous site, direct competition for the same site, or indirect competition for a necessary binding component. The disclosure of site II indicates that this site may be physically removed and (or) biochemically distinct from site I.

Table 1 compares the values for the affinity and maximum binding capacity obtained by Ligand analysis of the individual Scatchard plots. Both the experimental (+Con A) and the theoretical (–Con A) binding values for site II mathematically approximate the same line. The affinity reported here cannot be directly compared with published values of average affinity constants which are based on receptor occupancy and the negative cooperativity model⁶. The maximum binding capacity, used to determine the number of sites per cell, varies considerably in the literature depending on the highest concentrations of ligand chosen by the investigator for extrapolation of the Scatchard plot to provide an abscissa intercept; for example, reported values for erythrocytes vary from 34 (ref. 10) to 2,000 (ref. 1) sites per cell. There are approximately 11 sites per cell calculated from the R2 value in Table 1, and R1 represents an average of approximately one site per cell; yielding a total of 12 insulin binding sites per erythrocyte when analysed by a two-site model. In view of these data, the difficulty in documenting a physiological response to insulin in erythrocytes is understandable.

Experimental parameters investigated in these experiments describe receptor characteristics well within the normal physiological range of circulating insulin concentrations (0.2 – 20 ng ml⁻¹ = 5 – 475 μ U ml⁻¹). As insulin concentrations are increased beyond this range, experimental data demonstrate that trace amounts of labelled insulin continue to be bound, accounting for the asymptotic nature of the Scatchard plot. This nonsaturable component can be treated mathematically by the Ligand program.

Erythrocytes demonstrate both enhanced dissociation¹ and, as shown here, heterogeneous populations of receptors. Enhanced dissociation of ¹²⁵I-insulin from receptors in the presence of excess unlabelled insulin has been explained by negative cooperativity between homogeneous receptors⁶. However, the phenomenon of enhanced dissociation cannot be explained by the presence of non-interacting, heterogeneous sites¹¹. It is thus possible that there are site-site interactions among receptors of a single type, as suggested for adipocyte insulin receptors¹², or that one receptor population consists of an aggregated form of the other receptor population.

There are several interesting parallels between membrane receptors for Con A and insulin. Con A erythrocyte receptors are associated with physical particles on the membrane which influence glucose flux¹³, and a specific minor membrane glycoprotein known to influence anion permeability¹⁴. Both of

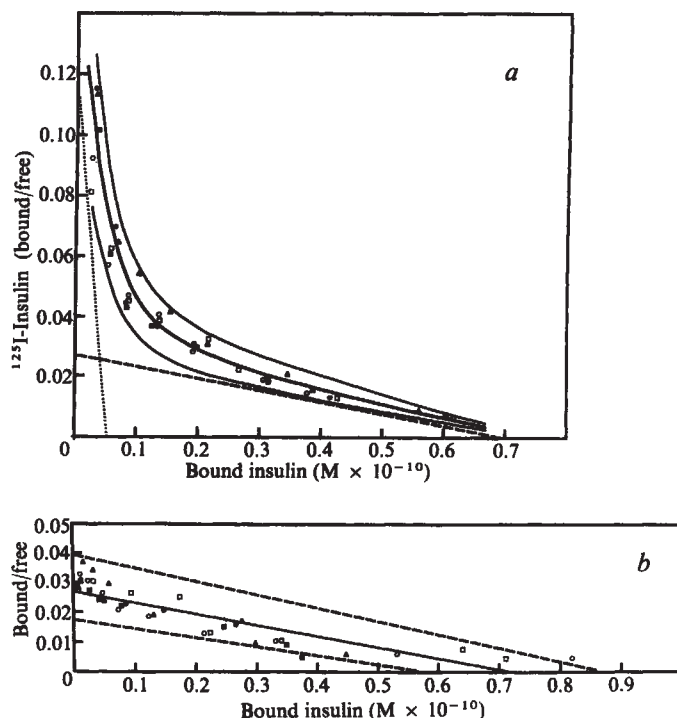


Fig. 2 Scatchard plots of insulin binding to erythrocytes from five individuals (as in Fig. 1) in the absence (a) and presence (b) of Con A. The ratio of bound to free insulin is plotted as a function of the molar quantity of bound insulin. Insulin concentrations varied from 0.2 to 100 ng ml⁻¹ in the absence of Con A and from 0.2 to 20 ng ml⁻¹ in the presence of Con A. a, Computer resolution of the curvilinear Scatchard plot, shown as the best fit line ± 2 s.d. yields high affinity (.....) and low affinity (----) components. b, Data points obtained in the presence of Con A (5 – 10 μ g ml⁻¹) are shown in comparison to receptor site II (—) ± 2 s.d. (----) as determined by computer analysis of data in the absence of Con A.

these transmembrane functions are stimulated by insulin in hormone-sensitive cell types. Insulin has also been shown to interfere with Con A binding sites on myoblasts, thus inhibiting the Con A-stimulated formation of myotubules¹⁵. Although these observations seem to favour a common or physically adjacent site for Con A and insulin membrane receptors, it is also possible that these molecules compete for the component(s) necessary for aggregation of receptor sites, a phenomenon observed for both Con A¹⁴ and insulin¹⁸ receptors. The data reported here augment these important similarities between Con A and insulin receptors and also provide evidence for heterogeneous populations of insulin receptors on human erythrocytes.

We thank Dr Dan Sheehan for verifying the original Rosenthal analyses, Drs David Rodbard and Peter Munson for helping us implement the 'Ligand' program; Drs David Straub and Jim Norris for reviewing the manuscript, and Dr Sanford Roth for initial suggestions. This work was supported by institutional grants from the University of Arkansas for Medical Sciences.

Received 31 January; accepted 16 April 1980.

- Gambhir, K. K., Archer, J. A. & Bradley, C. J. *Diabetes* **27**, 701–708 (1978).
- Wachslight-Rodbard, H., Gross, H. A., Rodbard, D., Ebert, M. H. & Roth, J. *New Engl. J. Med.* **300**, 882–887 (1979).
- Robinson, T. J. *et al. Science* **205**, 200–202 (1979).
- Scatchard, G. *Ann. N.Y. Acad. Sci.* **51**, 660–672 (1949).
- Gavin, J. R. III, Gordon, P., Roth, J., Archer, J. A. & Buell, D. N. *J. biol. Chem.* **248**, 2202–2207 (1973).
- De Meyts, P., Bianco, A. R. & Roth, J. *J. biol. Chem.* **251**, 1877–1888 (1976).
- Cuatrecasas, P. *J. biol. Chem.* **248**, 3528–3534 (1973).

Table 1 Comparison of binding parameters between Scatchard plots in the presence and absence of Con A

	Site I		Site II	
	K_1 $\times 10^{10}$ M ⁻¹	R_1 $\times 10^{-12}$ M	K_2 $\times 10^8$ M ⁻¹	R_2 $\times 10^{-11}$ M
No Con A*	2.25 ± 1.07	5.39 ± 1.6	3.83 ± 0.81	7.11 ± 0.72
+Con A†	Undetectable		3.95 ± 0.56	7.03 ± 0.90

The values for affinity (K) and maximum binding (R) were determined from data illustrated in Fig. 2 and averaged to determine the mean $\pm$ s.e. (n = 5).

*From experimental data points shown in Fig. 2a.

†From experimental data points shown in Fig. 2b.

8. Munson, P. J. & Rodbard, D. *Analyt. Biochem.* (in the press).
9. Rosenthal, H. E. *Analyt. Biochem.* **20**, 525-532 (1967).
10. Herzberg, V. L., Boughter, J. M., Carlisle, S. K., Ahmad, F. & Hill, D. E. *Pediat. Res.* **14**, 4-7 (1980).
11. Rodbard, D. *Am. J. Physiol.* **237**, E203-E205 (1979).
12. Olefsky, J. M. & Chang, H. *Diabetes* **27**, 946-958 (1978).
13. Pinto da Silva, P. & Nicolson, G. L. *Biochim. biophys. Acta* **363**, 311-319 (1974).
14. Gordon, J. A., Staehelin, L. A. & Kuettnner, C. A. *Expl Cell Res.* **110**, 439-448 (1977).
15. Kahn, C. R., Baird, K. L., Jarrett, D. B. & Flier, J. S. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4209-4213 (1978).
16. Freychet, P., Roth, J. & Neville, D. M. *Biochem. biophys. Res. Commun.* **43**, 400-408 (1971).
17. Schneider, B., Straus, E. & Yalow, R. *Diabetes* **25**, 260-267 (1976).
18. Sandra, A., Leon, M.A. & Przybylski, R. J. *Endocrinology* **105**, 391-401 (1979).

An effect of chloride on (Na + K) co-transport in human red blood cells

A. R. Chipfield

Department of Physiology, University of Dundee,
Dundee DD1 4HN, UK

In certain conditions, internal K can stimulate active Na:K transport as well as Na:Na exchange through the ouabain-sensitive Na, K exchange pump¹. When the exchange was examined during a study of the action of internal K, it emerged that the removal of K_o (the main requirement for Na:Na exchange^{2,3}) reduced the ouabain-insensitive Na efflux. Thus, in eight experiments done variously with outdated cells or high and low [K]_i, PCMBs-treated cells, the K_o-dependent Na efflux ranged from 0.18 to 0.70 μ Eq/ml cells per h. This conflicts with the well established view that the K_o-dependent and ouabain-sensitive components of Na efflux are identical^{3,4}. However, the K_o-dependent, ouabain-insensitive Na efflux was found to go through the (Na + K) co-transport system, which was further found to show a dependence on chloride ions.

In a further eight experiments, specifically designed to test the effect of K_o on the ouabain-insensitive Na efflux, the same pattern emerged. The K_o-dependent, ouabain-insensitive efflux varied from 0.16 to 0.96 μ Eq/ml cells per h (or from 8 to 41% of the total). But why did this happen in this study and not before? The reason seems to be because Ca, Mg and phosphate are absent in the external medium (this unusual feature derives from an earlier study of the kinetics of the pump⁵ where any possible interference was to be avoided; this does not affect the sensitivity to ouabain). Thus, when any of the ions Ca, Mg or phosphate were present the effect of K_o was reduced and with all three present it was abolished. Moreover, it was not seen when Na_o was replaced by Li or by

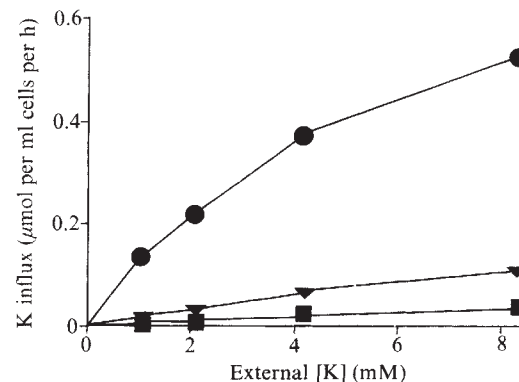


Fig. 1 External [K] dependence of ouabain-insensitive K influx into human RBC. K influx was measured as described in Table 2 (experiment 2): each point is the mean of two determinations (errors, which are not shown were comparable to those in Table 2). ●, Control: 150 mM [Cl]_o; ▼, 30 mM [Cl]_o; ■, 150 mM [Cl]_o with 1 mM furosemide (the results for 30 mM [Cl]_o with 1 mM furosemide (not shown) were the same as for 150 mM with furosemide).

Mg. Thus, there may be several reasons why a K_o-dependent, ouabain-insensitive Na efflux has not been seen before.

If there is a ouabain-insensitive, K_o-dependent component of Na efflux, it is most likely to be related to the furosemide-sensitive (Na + K) co-transport system identified in RBC by Wiley and Cooper⁶. If so, it is related to the furosemide-sensitive, external Cl-dependent K:K exchange in ascites cells^{7,8}. The results of one of five experiments designed to test these possibilities are shown in Table 1. Three features were noted: (1) furosemide inhibited the ouabain-insensitive, K_o-dependent efflux, (2) on replacing most of the Cl_o with nitrate, Na efflux was reduced and (3) the ouabain-insensitive, K_o-dependent efflux was smaller in nitrate-containing media.

These results, taken together with the studies mentioned before⁶⁻⁸, are consistent with the idea that a large part of the Na efflux goes by a (Na + K + Cl) co-transport system. If this is so, then the furosemide-sensitive K influx should be reduced on lowering [Cl]_o. The results of the first experiment in Table 2 (leaving aside, for the moment, the tests with phloretin) bear out this prediction.

The results of the second experiment in Table 2 show that similar results were obtained when K influx was assessed using ⁸⁶Rb as tracer (this was also true for Rb influx). Accordingly, this technique was used in further comparisons with earlier work. Thus, the saturable, furosemide-sensitive passive K influx^{2,3,6} was reduced on lowering [Cl]_o (Fig. 1). There was a roughly linear dependence on [Cl]_o (ref. 8) of K influx (Fig. 2). With anions other than nitrate, there was a substantial furosemide-sensitive K influx with bromide but not with

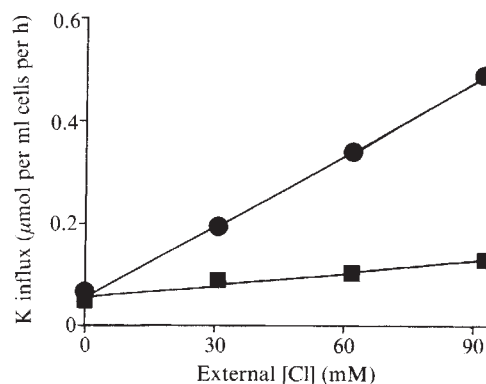


Fig. 2 External [Cl] dependence of ouabain-insensitive K influx into human RBC. K influx was measured as described in Table 2 (experiment 2): each point is the mean of three determinations (errors (not shown) were comparable to those in Table 2). ●, Control; ■, with 1 mM furosemide.

Table 1 Tracer ²⁴Na efflux from human RBC in the presence of ouabain

External medium	Na efflux (μ Eq/ml cells per h)	
	With 150 mM [Cl] _o	With 30 mM [Cl] _o
Complete	2.19 $\pm$ 0.31	1.73 $\pm$ 0.12
K-free	1.74 $\pm$ 0.14	1.78 $\pm$ 0.14
Complete + furosemide (1 mM)	1.48 $\pm$ 0.12	1.59 $\pm$ 0.14
K free + furosemide (1 mM)	1.65 $\pm$ 0.17	1.65 $\pm$ 0.14

Tracer ²⁴Na efflux from outdated human RBC was measured as described elsewhere¹⁻⁵ in a medium containing 142 mM NaCl, 8 mM KCl, 8 mM Tris Cl, pH 7.5, 10 mM inosine¹⁶ and 0.1 mM ouabain. When K was omitted, KCl was replaced by NaCl: external [Cl] was reduced by replacing NaCl, KCl and Tris-Cl by the corresponding nitrates. The results of this and the other four similar experiments show that the K_o-dependent, ouabain-insensitive flux in 150 mM external chloride is statistically significant (Student's *t* test, *P* < 0.003); in contrast, this component of Na influx is not significant in media containing 30 mM [Cl]_o, furosemide or both. Results are the means of three determinations ($\pm$ s.e.m.).

Table 2 K influx into human RBC in the presence of ouabain

External medium	Experiment 1		Experiment 2	
	K influx (tracer ^{42}K) (μEq per ml cells per h)		K influx (tracer ^{86}Rb) (μEq per ml cells per h)	
	Control	With furosemide	Control	With furosemide
Complete	0.83 ± 0.04	0.26 ± 0.00	0.99 ± 0.00	0.25 ± 0.00
30 mM $[\text{Cl}]$	0.36 ± 0.01	0.25 ± 0.02	0.36 ± 0.05	0.29 ± 0.01
Complete + phloretin	0.46 ± 0.04	0.24 ± 0.03	0.64 ± 0.01	0.24 ± 0.00
30 mM $[\text{Cl}]$ + phloretin	—	—	0.28 ± 0.01	0.24 ± 0.02

K influx into outdated human RBC was measured as described elsewhere¹⁻⁵ in the same conditions (and with the same changes) as those given in Table 1; the concentration of phloretin used was 0.1 mM. The results shown are the mean of three determinations ($\pm$ s.d.).

sulphate or acetate: bicarbonate raised both the furosemide-sensitive and furosemide-insensitive components of K influx. These new observations on cation and anion affinities fit the previous findings^{2,3,6,8}. It was also shown that passive K influx was half-maximally inhibited by 5×10^{-6} M furosemide.

Three other sets of results should be mentioned. First, experiments on fresh cells: here, the effects of furosemide and $[\text{Cl}]_0$ reduction on Na efflux were the same but K_0 had no effect (giving another reason why this flux has not been seen before). Second, experiments with phloretin: this drug reduced, but did not abolish, the K_0 - and Cl_0 -dependent Na efflux and likewise for K influx (Table 2). Moreover, furosemide and phloretin together inhibited no more than furosemide alone. Evidently, the co-transport system is the same as the phloretin- and furosemide-sensitive (Na-Li) counter-transport system identified in RBC by Tosteson and co-workers⁹. Third, there is the extensive work by Wieth on anion and cation effects on passive fluxes in RBC^{10,11}: some of his results, for example, an increase in Na influx in 120 mM nitrate, seem to conflict with mine. The most likely explanation for these discrepancies is that his media contained Ca, Mg and phosphate, all of which may alter the results (see above) as well as bicarbonate, which alters passive permeability too¹⁰.

(Na+K) co-transport⁶ is clearly affected by chloride but direct evidence for Cl^- transport by the same system in RBC is lacking. On the other hand, furosemide-sensitive co-transport (or counter-transport) for each pair of ions has been found in several tissues and often these are invoked to explain ion distributions that do not seem to be in equilibrium^{6-9,12-14}. Is it reasonable to speculate that some of these share the same pathway? Certainly, this view would be consistent with the idea that 'dissipative' fluxes may be coupled to uphill movements of another species¹⁵.

I thank Mrs C. J. Hall for technical assistance, the Scottish Hospitals Endowments Research Trust for financial support and Hoechst UK Ltd for the gift of furosemide.

Note added in proof: A recent, independent study by Dunham *et al.*¹⁷ also bears on the chloride dependence of furosemide-sensitive fluxes.

Received 13 September 1979; accepted 21 April 1980.

- Garay, R. P. & Garrahan, P. J. *J. Physiol., Lond.* **231**, 297-325 (1973).
- Garrahan, P. J. & Glynn, I. M. *J. Physiol., Lond.* **192**, 189-216 (1967).
- Glynn, I. M., Lew, V. L. & Luthi, U. *J. Physiol., Lond.* **207**, 371-391 (1970).
- Glynn, I. M. *J. Physiol., Lond.* **136**, 148-173 (1957).
- Chipperfield, A. R. & Whittam, R. J. *J. Physiol., Lond.* **260**, 371-385 (1976).
- Wiley, J. S. & Cooper, R. A. *J. clin. Invest.* **53**, 245-255 (1974).
- Tupper, J. T. *Biochim. biophys. Acta* **394**, 586-596 (1975).
- Bakker-Grunwald, T. *Biochim. biophys. Acta* **513**, 292-295 (1978).
- Pandey, G. N. *et al.* *J. gen. Physiol.* **72**, 233-247 (1978).
- Funder, J. & Weith, J. O. *Acta physiol. scand.* **71**, 168-185 (1967).
- Weith, J. O. *Acta physiol. scand.* **79**, 76-87 (1970).
- Heinz, E., Geck, P., Pietrzyk, C., Burckhardt, G. & Pfeiffer, B. *J. supramolec. Struct.* **6**, 125-133 (1977).
- Russell, J. M. *J. gen. Physiol.* **73**, 801-818 (1979).
- Eveloff, J., Hildmann, B., Kinne, R. & Murer, H. *J. Physiol., Lond.* **285**, 9P-10P (1978).
- Lew, V. L. & Beauge, L. in *Membrane Transport in Biology* Vol. 2 (eds Giebisch, G., Tosteson, D. C. & Ussing, H. H.) 81-115 (Springer, Berlin, 1979).
- Whittam, R. & Wiley, J. S. *J. Physiol., Lond.* **199**, 485-494 (1968).
- Dunham, P. B., Stewart, G. W. & Ellory, J. C. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1711 (1980).

High-affinity binding sites for juvenile hormone I in the larval integument of *Drosophila hydei*

Gerhard Klages & Hans Emmerich

Institut für Zoologie, Technische Hochschule Darmstadt, Schnittpahnstrasse 10, D 6100 Darmstadt, FRG

Martin G. Peter

Institut für Organische Chemie und Biochemie, Rheinische Friedrich-Wilhelms-Universität Bonn, Gerhard-Domagk-Strasse 1, D 5300 Bonn, FRG

Insect juvenile hormones (JH) maintain larval characteristics of insects during growth and development. At high JH titres the larval hypodermis is programmed to secrete larval cuticle in the following moult¹. Generally, the molecular events preceding the response of a target cell to a hormonal stimulus involve binding of the hormone to specific receptor molecules. There are little data available on the primary mechanism of action of JH at the molecular level. We have demonstrated the specific uptake and retention of JH-I in the cytosol of larval *Drosophila hydei* integument *in vitro* (G.K. and H.E., in preparation). We now report the discovery of high-affinity, high-specificity JH-binding principles that exhibit characteristic properties of hormone receptors, in the cytosol of early third instar *D. hydei* larval integument.

Because it has been established that receptors for steroid hormones in vertebrates² and invertebrates, especially the fruitfly *Drosophila*^{3,4} and the crayfish *Orconectes*⁵, exist in the cytosol of target cells in only very low concentrations, we started our search for receptors of the non-steroid JH using competition experiments with standard techniques. A large excess of nonradioactive racemic (2E,6E)-10,11-cis-JH-I was competed with either racemic or optically pure preparations of JH-I (ref. 6) labelled with tritium at high specific activity. The results are shown in Fig. 1: the presence of competitive (specific) JH-I binding sites becomes apparent from the competition curves for both racemic and optically active preparations of radiolabelled JH-I. However, remarkably different results were obtained; first, the enantiomerically pure, naturally occurring (10R,11S)-JH-I is bound to cytosol

Table 1 Competitive displacement of [^3H](10R,11S)-JH-I from partially purified high-affinity JH-binding sites by enantiomerically pure (10R,11S)-JH-I and racemic JH-I, respectively

Competitor (ng per test tube)	% Displacement of [^3H](10R,11S)-JH-I by	
	(10R,11S)-JH-I	Racemic JH-I
0.4	24.7	1.7
1.3	48.8	10.0
9.1	54.8	20.9

High-affinity JH-binding sites were prepared by gel filtration of cytosol through Sepharose 6-B, equilibrated with TMG-buffer, pH 7.2, at 0°C. The specific JH-binding sites were contaminated with nonspecific JH-binding sites. 8,600 c.p.m. [^3H](10R,11S)-JH-I (0.32 ng per test tube = 1.1 nM) were incubated for 1 h at 4°C with the indicated concentrations of (10R,11S)-JH-I or racemic JH-I. Bound radioactivity was measured in 100- μl aliquots by the charcoal adsorption technique. The enantiomerically pure (10R,11S)-JH-I was extracted from 3-4 day old *Hyalophora cecropia* accessory glands. The purification method of extracts includes low temperature precipitation of lipids, TLC on silica gel, chromatography on Sephadex LH-20, and HPLC on LiChrosorb Si 60 and Si 100. Purity was controlled by analytical HPLC on LiChrosorb Si 100.

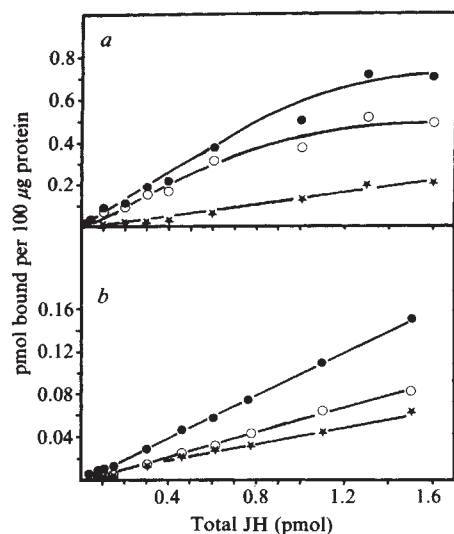


Fig. 1 Binding of *a*, enantiomerically pure [*methoxy*-³H] (10*R*,11*S*)-JH-I (specific activity ~58 Ci mmol⁻¹ (ref. 6)) and *b*, racemic [¹⁰⁻³H]-JH-I (specific activity 11.5 Ci mmol⁻¹, NEN) to cytosol components. Larval integuments from early third instar larvae (120h) were prepared and homogenized as described elsewhere¹⁴. Cytosol (120,000*g* supernatant) was prepared in TMG-buffer, pH 7.2, consisting of 10 mM Tris(hydroxy-methyl)-aminomethane, 15 mM β-mercaptoethanol, 20% glycerol, 1 mM EDTA, 50 mM KCl (all from Merck, Darmstadt), and 0.43 mM Trasylol (Bayer). JH metabolism was inhibited with 1 mM *p*-hydroxymercuribenzoate (Serva)¹⁴. Samples (100 µl) (containing 80–120 µg protein as determined in aliquots by the Lowry method¹⁵) of the cytosol were equilibrated for 30 min at 4°C with increasing concentrations of [*methoxy*-³H] (10*R*,11*S*)-JH-I (0.02–1.6 pmol per 100 µl sample) or racemic [¹⁰⁻³H]-JH-I (0.04–1.5 pmol per 100 µl sample) in the absence (●) and presence (★) of a 100-fold excess of unlabelled racemic JH-I (Eco-Control), respectively. Bound and free radioactivity was separated by the charcoal adsorption technique with 0.42% dextran-coated charcoal (Serva)⁸. 100 µl of the supernatant (bound radioactivity) were measured by liquid scintillation counting using Aqualuma (Baker) as scintillation cocktail. The means of two duplicates, normalized on the basis of 100 µg cytosol protein are plotted. Specific binding (○) was considered as difference of total binding (●) and nonspecific binding (★).

constituents with a higher affinity than the racemic JH-I, even if the possibility is considered that 50% of the latter, represented by the (10*S*,11*R*) enantiomer, which does not occur naturally, is not bound at all. Second, in the concentration range tested, only the tritiated (10*R*,11*S*) enantiomer seems to be able to saturate the specific JH binding sites of the cytosol completely. It may be important to consider that with 1.6 pmol JH-I per test tube the limit of solubility is nearly reached in our buffer system on addition of the excess unlabelled JH-I. Third, the ratio of specific to nonspecific JH binding sites seems to depend on the optical purity of the hormone tested. Using tritiated (10*R*,11*S*)-JH-I it is 2.5, whereas it is only 1.5 using tritiated racemic JH-I.

Equilibrium dissociation constants (K_D) of JH binding sites were first determined in equilibrium conditions. Figure 2 shows the Scatchard⁷ plots of binding data obtained with [*methoxy*-³H] (10*R*,11*S*)-JH-I and with racemic [¹⁰⁻³H]-JH-I. Increasing concentrations of the enantiomerically pure tritiated (10*R*,11*S*)-JH-I reveal the presence of two binding components with different affinities for the JH-I molecule. The apparent equilibrium dissociation constant (K_D) for the high-affinity JH-binding sites is 3.6×10^{-9} M. Similar equilibrium dissociation constants have been described for the macromolecular binding sites for ecdysteroids in *D. melanogaster* imaginal disks³ and K_c cell lines⁴. The second JH-binding component exhibits a much lower affinity for JH-I ($K_D \approx 10^{-7}$ – 10^{-6} M). Normalizing the data on the basis of protein content in the

cytosol and correcting for low-affinity binding sites, a concentration of 7.7 pmol per mg cytosol protein is calculated for the free high-affinity JH-binding sites. With tritiated racemic JH-I (Fig. 2, inset), four JH-binding components are detectable. One of them shows a similar K_D value (4×10^{-9} M) to the high-affinity binding sites, detected with the enantiomerically pure JH-I. The additional binding components show apparent K_D values of 2×10^{-7} M, 8×10^{-7} M, and 10^{-5} M, with one of them ($K_D = 2 \times 10^{-7}$ M) corresponding well to the haemolymph carrier protein ($K_D = 1 \times 10^{-7}$ M)⁸. As it is essential to clarify whether this component results from contamination of the tissue preparation with haemolymph or whether it truly represents an intracellular constituent, we injected third instar larvae with ¹⁴C-inulin solution. Following preparation of integuments 30 min after injection, the amount of label present was 0.65% of the total administered. Taking the concentration of the free JH-carrier protein into account⁸, it is thus established that less than 4% of the 2×10^{-7} M JH-binding component found in the cytosol preparation results from contamination with haemolymph. It is therefore of interest first to isolate and characterize this JH-binding component for comparative studies with the haemolymph JH-carrier protein, and second, to investigate the possible role of the JH-carrier protein in the process of JH uptake into target tissues. An interesting analogy might be represented in this context by the mechanism of uptake of the diterpenoids retinol and retinoic acid in vertebrate target tissues⁹.

An additional method was applied to obtain K_D values by measuring association and dissociation kinetics at 4°C, using racemic [¹⁰⁻³H]-JH-I. The association of JH-I with cytosol constituents, shown in Fig. 3, follows second-order kinetics. Equilibrium between association and dissociation is reached after 30 min. Transformation of binding data into linear form (Fig. 3, inset) yields the association rate constant (k_{+1}) for total JH-binding sites. From the slope of the straight line, a k_{+1} of 2×10^6 M⁻¹ × min⁻¹ is calculated. After reaching equilibrium the cytosol was chased with a 500-fold excess of non-labelled racemic JH-I. At appropriate times, bound radioactivity was measured in cytosol aliquots. From those data (not shown) a half-life time of approximately 8 h at 4°C for the hormone-macromolecule complex and a dissociation rate constant (k_{-1}) of 6.5×10^{-4} min⁻¹ was calculated. The kinetic data are not impaired by a measurable loss of binding activity during the incubation period, due to the pronounced stability of the competitive JH-binding sites at low temperatures; even after maintaining the cytosol for 2 days in

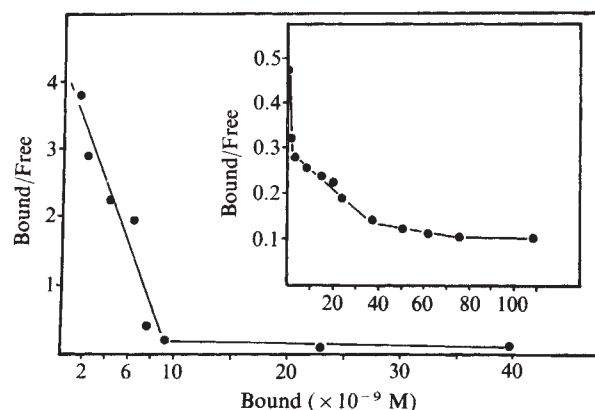


Fig. 2 Scatchard⁷ analysis of ³H-JH-I binding to cytosol components. Samples (100 µl) of larval integument cytosol, prepared as in Fig. 1, were equilibrated for 30 min at 4°C with increasing concentrations of [*methoxy*-³H] (10*R*,11*S*)-JH-I (2–300 nM) or racemic [¹⁰⁻³H]-JH-I (1–1,100 nM) (inset), respectively. Bound radioactivity was measured as in Fig. 1. Each point represents the mean of duplicate experiments. Repeat experiments gave similar results.

ice, no change in binding behaviour was detectable. From the kinetic data an equilibrium dissociation constant ($k_{-1}/k_{+1} = K_D$) of 3.2×10^{-10} M results for the JH-binding sites, which is one order of magnitude lower than that obtained from the equilibrium binding data. Such differences are not uncommon in studies with protein-ligand complexes¹⁰. In any case, the K_D values obtained from both kinetic and equilibrium data clearly prove the presence of high-affinity JH-binding sites in the cytosol of *D. hydei* integument.

We do not know the biological significance of JH-binding sites showing different affinities for the JH molecule. We believe that the high-affinity binding sites might serve as JH receptors in the cytosol, comparable to those described for steroid hormones. Further support for this assumption is presented by experimental approaches towards this binding component. A molecule serving as JH receptor should meet the following requirements:

(1) receptors must be macromolecules. Gel filtration of crude cytosol preparations on Sephadex G-25 and on Sepharose 6-B, equilibrated with TMG-buffer, pH 7.2, without glycerol, revealed the macromolecular nature of specific JH-binding sites. Heating the macromolecular cytosol fractions for 10 min at 60°C completely destroys the competitive binding activity. (2) Receptors must show a high specificity for the natural hormone. The potential of some JH homologues and analogues to compete with [*methoxy*-³H](10*R*,11*S*)-JH-I and racemic [³H]JH-I for JH-binding sites was studied with crude cytosol. Among the compounds tested, racemic JH-I and JH-III were the most effective competitors of [*methoxy*-³H](10*R*,11*S*)-JH-I and racemic [³H]JH-I bound to cytosol components (Fig. 4). The capacity to compete with the binding of [*methoxy*-³H](10*R*,11*S*)-JH-I decreases in the following order: JH-I > JH-III > ethyl ester analogue of JH-III > methyl farnesoate > ZR515. The sequence of competition efficiency with [³H]JH-I is: JH-I ≈ JH-III > ethyl ester analogue of JH-III > ZR515 > methyl farnesoate. Alterations in the structure of the JH-III molecule (the absence of the epoxy group as in methyl farnesoate or the presence of a ethyl ester group instead of a methyl ester group) markedly reduce the competing efficiency. It is of interest that the highly potent insect growth regulator ZR515, which has been used in pest control¹¹, indeed competes only very poorly with the JH-binding sites.

Enantioselectivity as another means of determining the specificity of JH-binding sites was tested in partially purified preparations of high-affinity JH-binding sites. Table 1 shows

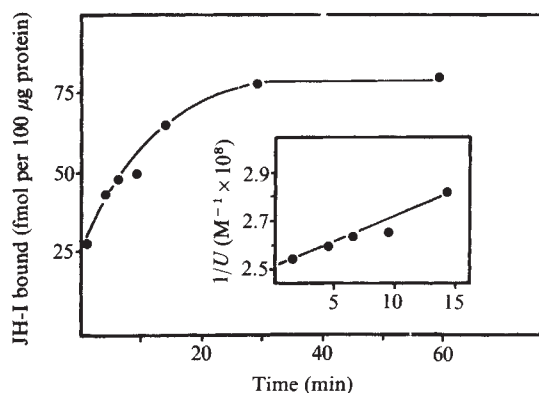


Fig. 3 Association kinetics of racemic [³H]JH-I binding to cytosol components. Samples (100 µl) of larval integument cytosol, prepared as in Fig. 1, were incubated with 4 nM racemic [³H]JH-I at 4°C for the incubation periods indicated and bound radioactivity was measured as stated above. The means of two duplicates, normalized on the basis of 100 µg protein, are plotted. Inset, the inverse of unbound hormone concentration ($1/U$) is plotted as a function of time. The association rate constant (k_{+1}) for total JH-I binding is calculated from the slope of the straight line.

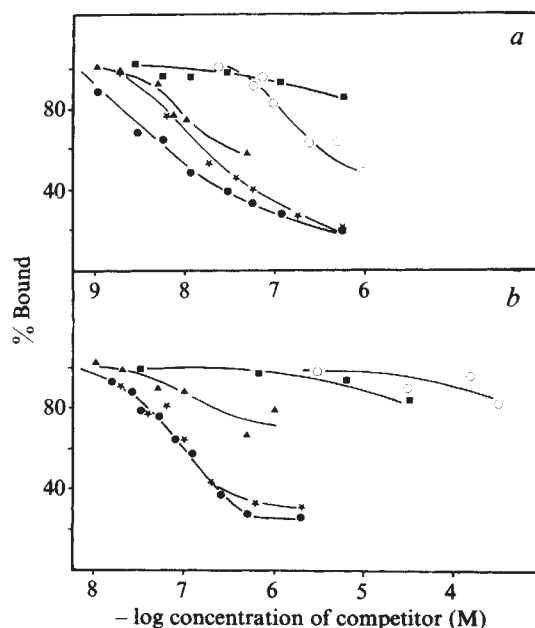


Fig. 4 Specificity of JH-binding sites in the cytosol of early third instar larvae integument. Samples (100 µl) of the cytosol, prepared as in Fig. 1, were equilibrated for 30 min at 4°C with: a, 1.1 nM [*methoxy*-³H](10*R*,11*S*)-JH-I (8,600 c.p.m. specific activity ~ 70 Ci mmol^{-1}) or b, 6.5 nM racemic [³H]JH-I (8,600 c.p.m., specific activity 11.5 Ci mmol^{-1}) and increasing concentrations of competitors. Bound radioactivity was measured as stated above. Each point represents the mean of two to four duplicates. Tritiated JHs and unlabelled competitors were purified by HPLC on Porasil columns (Waters Associates) with *n*-hexane/8% ethyl acetate or 8% diethylether as solvent system and/or by TLC on silica gel plates with benzene/ethyl acetate (7:1). ZR515 (Zoecon) was 96.8% pure. ●, JH-I (methyl-2*E*,6*E*-*cis*-3,11-dimethyl-7-ethyl-10,11-epoxy-2,6-tridecadienoate) ★, JH-III (methyl-2*E*,6*E*-3,7,11-trimethyl-10,11-epoxy-2,6-dodecadienoate); ▲, (JH-III-E, ethyl-2*E*,6*E*-3,7,11-trimethyl-10,11-epoxy-2,6-dodecadienoate (a gift of H. Rembold, MPI f. Biochemie, Martinsried)); ○, FME (methyl-2*E*/Z,6*E*/Z-3,7,11-trimethyl-2,6,10-tridecadienoate); ■, ZR515, (isopropyl-2*E*,4*E*-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate).

that optically pure unlabelled (10*R*,11*S*)-JH-I competes with binding of [*methoxy*-³H](10*R*,11*S*)-JH-I better than the racemic unlabelled JH-I, thus demonstrating enantioselective JH binding to the high-affinity JH-binding sites. Similar results have been reported previously for JH-specific haemolymph carrier proteins of *Manduca sexta*¹² and *Locusta migratoria*¹³. Surprisingly, especially at low competitor concentrations the unlabelled naturally occurring (10*R*,11*S*) enantiomer competes with the tritiated (10*R*,11*S*) enantiomer much better than the theoretically expected factor 2, when compared with the racemate. Considering only the data of Fig. 1, it may be suggested that the specific activity of the two radioactive JH-I preparations would have to be determined more carefully. This point, however, is of minor importance in the competitive binding studies depicted in Table 1. Clearly, there is a difference in binding behaviour of the racemate and the optically active preparation, the magnitude of which cannot be explained with our rationale on simple stereochemical arguments. A factor of 3 has been observed for the difference of binding of racemic and optically active JH-III preparations to the JH-carrier protein from larval *M. sexta* haemolymph¹². Furthermore, in the *Galleria* bioassay, (10*R*)-JH-III is about three times more active than the racemate¹². Due to the lack of more conclusive data, we have to consider the possibility that presently unknown effects govern the binding of radiolabelled JHs to their receptors.

The experiments performed so far present unequivocal evidence that high-affinity, high-specificity receptor-type JH-I

binding sites are present in the cytosol of larval *D. hydei* integument. According to the general concept of hormone action, further work should analyse the interaction of these binding sites with other cellular components (nuclei) in order to investigate their physiological significance in more detail.

Received 20 December 1979; accepted 16 April 1980.

1. Riddiford, L. M. in *Invertebrate Tissue Culture, Applications in Medicine, Biology and Agriculture* (eds Kustak, E. & Maramorosch, K.) 213–222 (Publisher, town, 1976).
2. Jensen, E. V. & DeSombre, E. R. A. *Rev. Biochem.* **41**, 203–230 (1972).
3. Yund, M. A., King, D. S. & Fristrom, J. W. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6039–6043 (1978).
4. Maróy, P., Dennis, R., Beckers, C., Sage, B. & O'Connor, J. D. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6035–6038 (1978).
5. Kupfert, P., Wilhelm, S. & Spindler, K.-D. *J. comp. Physiol.* **128B**, 95–100 (1978).
6. Peter, M. G., Gunawan, S. & Emmerich, H. *Experientia* **35**, 1141–1142 (1979).
7. Scatchard, G. *Ann. N.Y. Acad. Sci.* **51**, 660–672 (1949).
8. Klages, G. & Emmerich, H. *Insect Biochem.* **9**, 23–30 (1979).
9. Chen, C.-C. & Heller, J. *J. biol. Chem.* **252**, 5216–5221 (1977).
10. Hansen, P. E., Johnson, A., Schrader, W. T. & O'Malley, B. W. *J. Steroid Biochem.* **7**, 723–732 (1976).
11. Henrick, C. A., Staal, G. B. & Siddall, J. B. *J. Agric. Fd Chem.* **21**, 352–359 (1973).
12. Schooley, D. A., Bergot, B. J., Goodman, W. & Gilbert, L. I. *Biochem. biophys. Res. Commun.* **81**, 743–749 (1978).
13. Peter, M. G., Gunawan, S., Gellissen, G. & Emmerich, H. *Z. Naturforsch.* **34c**, 588–598 (1979).
14. Klages, G. & Emmerich, H. *J. comp. Physiol.* **132**, 319–325 (1979).
15. Lowry, D. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265–275 (1951).

Molecular heterogeneity of benzodiazepine receptors

Werner Sieghart & Manfred Karobath

Department of Biochemical Psychiatry, University of Vienna, Lazarettgasse 14, A-1090 Vienna, Austria

Benzodiazepines exhibit reversible, stereospecific high affinity binding to mammalian brain membranes, and the respective binding sites for ^3H -flunitrazepam represent pharmacologically and clinically relevant receptors for benzodiazepines^{1–3}. Recently it has been demonstrated that reversibly bound ^3H -flunitrazepam becomes irreversibly attached to a specific membrane protein with apparent molecular weight of 50,000 when incubations were performed in the presence of UV light^{4,5}. Irreversible binding of ^3H -flunitrazepam to this protein had pharmacological properties similar to reversible benzodiazepine receptor binding, indicating that ^3H -flunitrazepam is a photoaffinity label for the benzodiazepine receptor⁵. Using irreversible binding of ^3H -flunitrazepam and subsequent electrophoretic separation of the labelled proteins in SDS-gels followed by fluorography⁶, we found that in hippocampus and several other brain regions at least two different types of benzodiazepine receptors exist. Each seems to be associated with a γ -aminobutyric acid (GABA) receptor.

Membranes from several rat brain regions were incubated with 10 nM ^3H -flunitrazepam at 0°C for 90 min, and the samples were then irradiated with UV light for 10 min. Membranes were collected by centrifugation, dissolved in SDS and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

^3H -flunitrazepam irreversibly binds to a single protein band with an apparent molecular weight of 51,000 (P_{51}) in cerebellum (Fig. 1). Similarly, most of the ^3H -radioactivity bound to membranes from other brain regions is associated with a protein band with the same apparent molecular weight. The extremely weak labelling of this protein band in spinal cord agrees with the very low specific reversible binding (data not shown) of ^3H -flunitrazepam to membranes from this tissue. Thus, P_{51} seems to be identical to the labelled benzodiazepine receptor described previously⁵. However, in other brain regions in addition to P_{51} several other protein bands with apparent molecular weights of 53,000 (P_{53}), 55,000

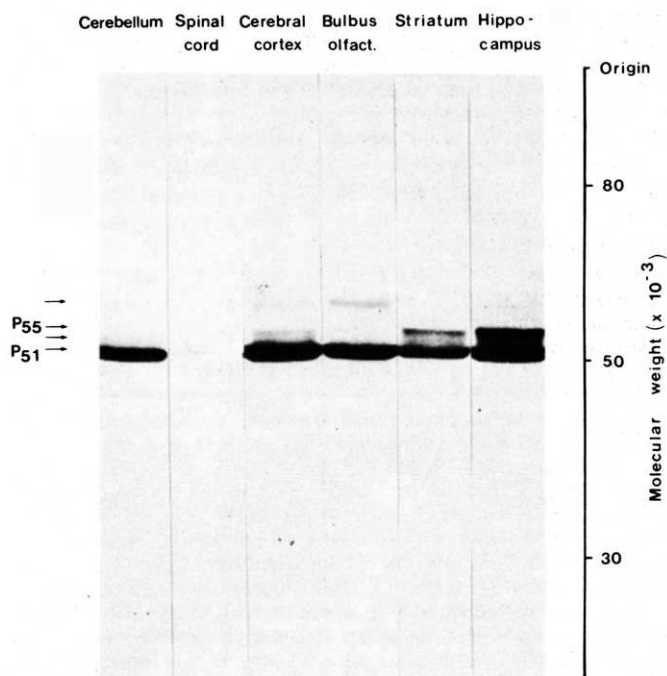


Fig. 1 Fluorography illustrating irreversible ^3H -flunitrazepam binding to membrane proteins from several rat brain regions. Tissues from several rat brain regions were homogenized with an Ultra Turrax for 30 s in 50 vol of 50 mM Tris-citrate buffer, pH 7.1, and centrifuged for 10 min at 48,000g. Pellets were washed four times by resuspension and recentrifugation in the same volume of buffer and stored at -20°C for at least 18 h. After thawing, aliquots of membranes derived from 6 mg tissue wet weight were incubated with 10 nM ^3H -flunitrazepam (84.3 Ci mmol^{-1} , NEN) in a solution containing 50 mM Tris-citrate buffer, pH 7.1, and 150 mM NaCl (ref. 9). Incubations were performed at 0°C for 90 min and the samples were then irradiated with UV light⁵ for 10 min. Membranes were centrifuged at 48,000g for 10 min, resuspended in 100 μl H_2O and 50 μl SDS stop solution (10% SDS (w/v), Tris HCl 100 mM, pH 7.4, β -mercaptoethanol 5 mM, sucrose 0.1 g ml^{-1} and bromophenol blue tracking dye 0.02 mg ml^{-1}) and placed in a boiling water bath for 5 min. Electrophoresis on 10% SDS-polyacrylamide slab gels and protein staining of the gels was performed as described previously¹⁰. After treatment with PPO, gels were dried and subjected to fluorography at -70°C on Kodak X-Omat R film⁶ for 6 days. All radioactive bands disappeared after incubation of the samples with pronase but not after three extractions with chloroform-methanol (3:1), and thus represent proteins. The apparent molecular weights of the labelled protein bands, indicated by the arrows (59,000: P_{59} ; 55,000: P_{55} ; 53,000: P_{53} and 51,000: P_{51}) were determined by calibrating the gel with standard proteins of known molecular weight. The pattern of bound radioactivity was unchanged in cerebellar or hippocampal membranes isolated and labelled in the presence of the protease inhibitor phenylmethylsulphonyl-fluoride (PMSF, 100 μM). Furthermore, co-homogenization of cerebellum and hippocampus and irreversible binding of ^3H -flunitrazepam to the resulting mixed membranes revealed a pattern of labelled protein bands which was additive and did not show any enhanced degradation of the hippocampal bands. This indicates that the heterogeneity of ^3H -flunitrazepam binding proteins cannot be explained by regional differences in the degradation of a single protein with higher molecular weight. As the efficiency of labelling of the different protein bands is not necessarily identical, no attempt was made to estimate the quantitative contribution of the various bands to irreversible benzodiazepine receptor binding.

(P_{55}) and 59,000 (P_{59}) are labelled with variable intensity. The most prominent of these additional bands is P_{55} which is most highly labelled in hippocampal membranes.

To characterize the pharmacological properties of irreversible ^3H -flunitrazepam binding to P_{51} and P_{55} , membranes from cerebellum and hippocampus were

Table 1 Influence of GABA and CL 218 872 on reversible and irreversible binding of ^3H -flunitrazepam to membranes or membrane proteins from rat cerebellum and hippocampus

	Specific ³ H-flunitrazepam binding (% of control)		
	Reversible	Irreversible	
		Membranes	Proteins
Cerebellum			
+10 μM GABA	200.7 ± 8.3	201.0 ± 0.2	251.7 ± 13.3
+1 μM CL 218 872	10.8 ± 1.5	16.9 ± 0.7	14.3 ± 0.4
Hippocampus			
+10 μM GABA	211.1 ± 2.9	198.8 ± 11.4	211.7 ± 11.1
+1 μM CL 218 872	45.9 ± 4.2	47.7 ± 0.2	49.9 ± 3.1

Cerebellar or hippocampal membranes were incubated with 0.4 nM ^3H -flunitrazepam either without additions (control) or in the presence of 10 μM GABA, 1 μM CL 218 872 or 2 μM diazepam as described in legend to Fig. 1. For the estimation of reversible binding, samples were then rapidly filtered as described previously⁹. For the estimation of irreversible binding to membranes samples were irradiated for 10 min with UV light after the 90 min equilibration period. In these conditions, irreversible binding of ^3H -flunitrazepam to membranes was about 30% of reversible binding. Exposure to UV light for a longer time period resulted in a higher percentage of irreversible binding but did not yield additional labelled protein bands. In this experiment the short irradiation time was selected because longer irradiation times change the kinetic properties of irreversible binding. After irradiation, 2 μM diazepam was added and samples were incubated at 30 °C for 60 min to remove non-irreversibly bound ^3H -flunitrazepam from membranes. Samples were then filtered and bound radioactivity was estimated as described previously⁹. For the estimation of irreversible binding to proteins, samples were treated as described in the legend to Fig. 2. As separation of the hippocampal proteins was not sufficient to allow for separate quantitation of the proteins in the gel system used, the region in the gel containing P_{51} to P_{55} was identified by fluorography, cut out and oxidized with a Packard Model 306 sample oxidizer to recover ^3H -radioactivity for liquid scintillation counting. Nonspecific binding (estimated in the presence of 2 μM diazepam) was subtracted from total binding to yield specific binding. Results are expressed as per cent of control binding (mean $\pm$ s.e.m.; $n=3$). Control binding given in d.p.m. per mg wet weight was 1,643 and 3,170 (reversible binding), 579 and 1,046 (irreversible binding to membranes), 264 and 533 (irreversible binding to proteins) for cerebellum and hippocampus, respectively.

investigated. As can be seen in Fig. 2, irreversible binding of ^3H -flunitrazepam to P_{51} in cerebellum and to P_{51} and P_{55} in hippocampus is completely inhibited by diazepam, a centrally active benzodiazepine. In contrast, binding to these proteins is not inhibited by Ro 5-4864, which selectively inhibits binding to peripheral benzodiazepine binding sites⁷ (data not shown). Furthermore, irreversible binding of ^3H -flunitrazepam to P_{51} in cerebellum and to P_{51} and P_{55} in hippocampus is stimulated by 10 μM GABA to a similar extent as is found with reversible binding to membranes from cerebellum or hippocampus (Fig. 2, Table 1). This stimulation by GABA is bicuculline sensitive (Fig. 2) indicating that GABA exerts its stimulatory effects on the labelling of the protein bands by way of a GABA receptor. These results indicate that irreversible binding of ^3H -flunitrazepam to P_{51} as well as that to P_{55} is similar to reversible benzodiazepine receptor binding. Thus, in different brain regions different types of benzodiazepine receptors may occur which are modulated by GABA receptors.

A heterogeneity of benzodiazepine receptors has been suggested previously when reversible benzodiazepine receptor binding was investigated in the presence of CL 218 872 (ref. 8). This substance, a triazolopyridazine, has anxiolytic but only weak sedative properties⁸. In contrast to benzodiazepines it inhibits reversible benzodiazepine binding with K_i values and Hill coefficients which differ in various brain regions⁸. We therefore investigated a possible differential effect of CL 218 872 on the irreversible binding of ^3H -flunitrazepam to the different binding proteins. Figure 2 shows that CL 218 872 inhibits irreversible binding of ^3H -flunitrazepam to P_{51} more strongly than binding to P_{55} . Such a selective effect can account for the different quantitative inhibition by CL 218 872 of reversible⁸ and irreversible binding of ^3H -flunitrazepam in cerebellum and hippocampus (Table 1). These results seem to indicate that P_{55} is or is part of a pharmacologically different type of benzodiazepine receptor which is unrelated to that which contains the P_{51} protein. Both of these receptors seem to be associated with a GABA receptor. It should be emphasised, however, that additional types of benzodiazepine receptors could exist since the role of P_{53} and P_{59} in benzodiazepine receptor binding has not yet been investigated. In addition, each labelled band could consist of more than one labelled protein which differ in structure and properties but not in apparent molecular weight.

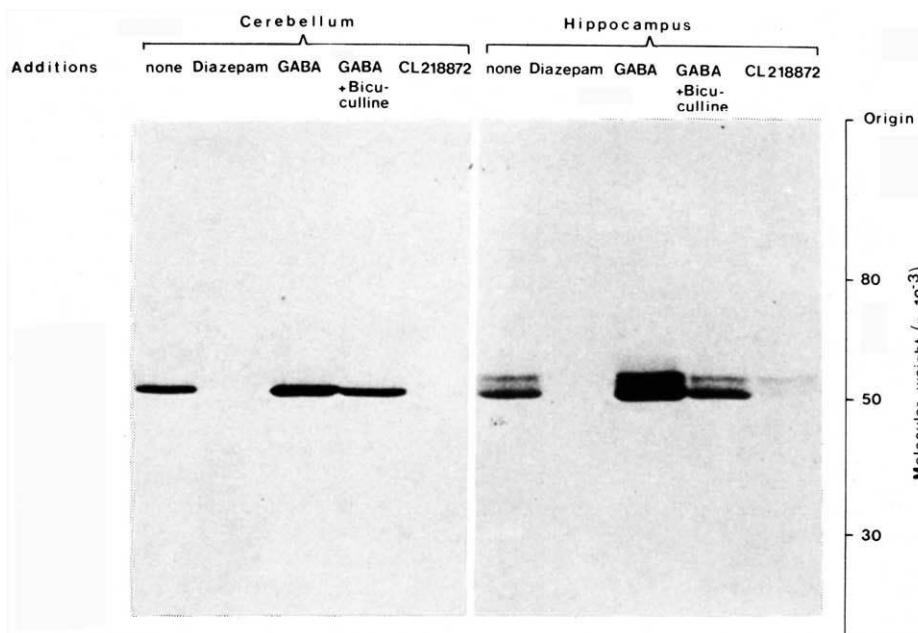


Fig. 2 Fluorography illustrating the influence of several drugs on irreversible ^3H -flunitrazepam binding to membrane proteins from cerebellum and hippocampus. Cerebellar or hippocampal membranes were incubated with 0.4 nM ^3H -flunitrazepam either without additions (lanes 1 and 6) or in the presence of 2 μM diazepam (lanes 2 and 7), 10 μM GABA (lanes 3 and 8), 10 μM GABA plus 10 μM bicuculline-methiodide (lanes 4 and 9), 1 μM CL 218 872 (lanes 5 and 10) or 1 μM Ro 5-4864 (experiment not shown). Incubation and irradiation of the samples as well as SDS-gel electrophoresis of the membrane proteins and fluorography of the gels was performed as described in the legend to Fig. 1.

Thus, this investigation indicates for the first time not only a pharmacological but also a molecular heterogeneity of benzodiazepine receptors. Using the present approach of irreversible labelling of benzodiazepine receptors followed by electrophoresis and fluorography, it seems possible to separate and isolate the different receptors. Furthermore, this technique can be used to develop new drugs which, in contrast to benzodiazepines, have selective effects on different types of benzodiazepine receptors.

We thank M. Forster for technical assistance. This work was supported by Fonds zur Förderung der wissenschaftlichen Forschung in Österreich.

Received 25 February; accepted 17 April 1980.

1. Möhler, H. & Okada, T. *Science* **198**, 849-851 (1977).
2. Squires, R. F. & Braestrup, C. *Nature* **266**, 732-734 (1977).
3. Speth, R. C., Wastek, G. J., Johnson, P. C. & Yamamura, H. I. *Life Sci.* **22**, 859-866 (1978).
4. Battersby, M. K., Richards, J. G. & Möhler, H. *Eur. J. Pharmac.* **57**, 277-278 (1979).
5. Möhler, H., Battersby, M. K. & Richards, J. G. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1666-1670 (1980).
6. Laskey, R. A. & Mills, A. D. *Eur. J. Biochem.* **56**, 335-341 (1975).
7. Braestrup, C. & Squires, R. F. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3805-3809 (1977).
8. Klepner, C. A., Lipka, A. S., Benson, D. I., Sano, M. C. & Beer, B. *Pharmac. biochem. Behav.* **11**, 457-462 (1979).
9. Karobath, M. & Sperk, G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1004-1006 (1979).
10. Krueger, B. K., Forn, J. & Greengard, P. *J. biol. Chem.* **252**, 2647-2773 (1977).

Axoplasmic transport of muscarinic receptors

Pierre Laduron

Department of Biochemical Pharmacology, Janssen Pharmaceutica, B-2340 Beerse, Belgium

The reality of axoplasmic transport is widely accepted; various neurotransmitters¹⁻³, enzymes^{2,4}, labelled proteins⁵ and peptides⁶ are known to move rapidly along the axons of different nerve fibres. In the terminals of sympathetic nerves, noradrenaline release is controlled by various regulatory mechanisms which imply the occurrence of presynaptic receptors⁷. In this regard, there is considerable indirect physiological evidence for the existence of muscarinic cholinergic receptors in the sympathetic nerve endings⁷⁻⁹; the stimulation by acetylcholine of such presynaptic receptors elicits an inhibitory effect on noradrenaline release^{8,9}. We now provide direct biochemical evidence for the occurrence in dog splenic nerve of muscarinic receptors which seem to move along the axon as suggested by their rapid accumulation on either side of a ligature.

Ligature experiments were carried out in mongrel dogs anaesthetized with pentobarbital (30 mg per kg, intravenously). Splenic nerve fibres were dissected from the splenic artery near the bifurcation, and a silk thread ligature was tied round the splenic nerve¹⁰. At 18 and 48 h, and 11 days after the operation, 1-cm segments of the nerve were removed, two above (proximal to) and below (distal to) the ligature. Coeliac ganglia were also removed and control splenic nerves were taken from unoperated dogs. After clearing connective tissue from the nerve fibres, the segments were weighed and then homogenized in 50 volumes of ice-cold distilled water by means of an ultraturax. The samples were further diluted to 1:200 with 0.05 M phosphate buffer, pH 7.4. Binding assays were carried out using 2 ml of tissue preparation with 2 nM ³H-dextetimide (specific activity 17.5 Ci mmol⁻¹, IRE Fleurus, Belgium) or 2 nM ³H-levetimide (specific activity 19 Ci mmol⁻¹, Janssen) as described previously²⁰. After incubation for 20 min at 37 °C, 3 ml of cold buffer was added and the samples were then rapidly filtered on Whatman GF/B glass filters. Stereospecific binding was taken as the difference between binding measured in the presence of 2 × 10⁻⁷ M levetimide (inactive enantiomer) and 2 × 10⁻⁷ M dextetimide (blank value).

To characterize the ³H-dextetimide binding sites in dog splenic nerves, various concentrations of dextetimide and levetimide were tested *in vitro* using a total homogenate. Figure 1 shows that dextetimide displaced the labelled ligand in the nanomolar range whereas levetimide was only effective in a micromolar concentration. This indicates the stereospecific nature of the binding which represents 50% of the total binding. Table 1 gives the IC₅₀ values for different compounds; atropine, an antimuscarinic drug which belongs to a different chemical class from dextetimide, also competed with ³H-dextetimide in the nanomolar range. Moreover, there was a good correlation between the IC₅₀ values of muscarinic antagonists, oxotremorine and levetimide, in dog splenic nerves and rat brain. As nicotine is not active, but antimuscarinic drugs and oxotremorine are, ³H-dextetimide binds in dog splenic nerves to receptors which have the characteristics of the muscarinic receptor. Further control experiments were carried out using ³H-levetimide as ligand; here, there was no stereospecific displacement and the value of the total binding corresponded to the blank values in the ³H-dextetimide binding. From these data, we conclude that the ³H-dextetimide binding sites in dog splenic nerves are of muscarinic nature.

Stereospecific ³H-dextetimide binding was measured in the proximal and distal part of dog splenic nerve at different times after ligature. Figure 1a shows that 18 h after constriction, the segments close to the ligature, P₁ and D₁, revealed more than a twofold increase in binding sites as compared with control nerves. In contrast, segments located 1 cm more proximally (P₂) or more distally (D₂) to the ligature and samples of the coeliac ganglion did not differ significantly from the control nerves. This accumulation in P₁ and D₁, so near the ligature, was still more pronounced 48 h after the operation (Fig. 2b). At that time the increase in binding sites was more than five times higher than control values in the proximal as well as in the distal part of the splenic nerves. This suggests a bidirectional transport of muscarinic receptors along the axon.

In contrast, the accumulation of noradrenaline was only detectable in the proximal segment, indicating a unidirectional transport for the biogenic amine, and confirming our earlier finding¹⁰. Interestingly, the increase of noradrenaline in the P₁ segment was of the same order of magnitude as the increase in muscarinic receptors measured in the binding assay. Here again, control experiments were carried out using ³H-levetimide as ligand; there was no increase in binding in the P₁ and D₁ segments even 48 h after ligature and again the total binding corresponded to the blank value of ³H-dextetimide binding.

To assess the origin of muscarinic receptors in the splenic nerves, ³H-dextetimide binding was assayed in proximal and

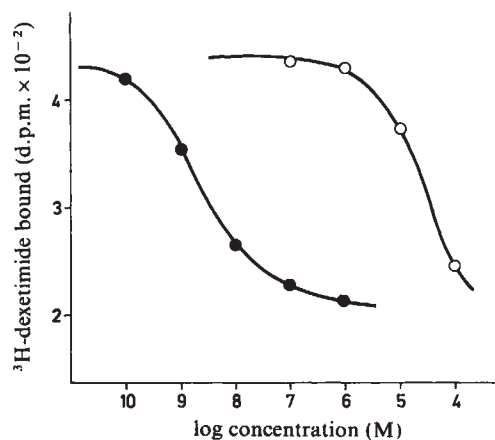


Fig. 1 Dose-response inhibition of dextetimide ((+)-benzetimide) (●) and levetimide ((-)-benzetimide) (○) expressed as -log concentration, in ³H-dextetimide assay using a total homogenate from dog splenic nerves.

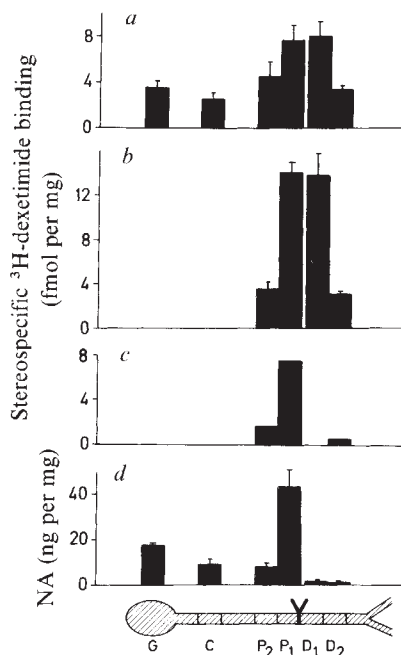


Fig. 2 Stereospecific ^3H -dextetimide binding and noradrenaline content in different segments of dog splenic nerves 18 h (a) 48 h (b, d) and 11 days (c) after ligature. Binding was assayed in duplicate; the results represent the mean ($\pm$ s.e.m.) obtained from five or six animals except for a ($n=3$) and d ($n=1$). Noradrenaline was measured according to the method of Coyle and Henry²¹. G=coeliac ganglion, C=control nerve from non-operated animals, P₁, P₂ and D₁, D₂ are, respectively, the proximal and distal part of ligated splenic nerves.

distal segments 11 days after ligature. Stereospecific binding sites were practically undetectable in the distal part, even in the segment closest to the constriction. In contrast, a clear-cut accumulation of binding sites remained detectable in the P₁ segment although to a lesser extent than that observed 48 h after the operation. This suggests that muscarinic receptors, like noradrenaline storage vesicles^{1,10,11}, originate from the perikarya of sympathetic neurones, in this case, in the coeliac ganglion.

The above results provide biochemical evidence for the presence of muscarinic cholinergic receptors in sympathetic nerves, giving more support to the concept of presynaptic or prejunctional receptors. In the heart, a substantial fraction of muscarinic receptors have been identified biochemically at the presynaptic sites in the sympathetic nerve terminals¹².

The most interesting finding here is the rapid accumulation in dog splenic nerves of muscarinic receptors both above and below a ligature, suggesting a bidirectional axonal flow for such binding sites. The fact that the rate of transport, about 1 mm h⁻¹, is practically the same as that previously found in similar conditions for noradrenaline storage vesicles, implies that muscarinic receptors are probably also associated with vesicle-like structures. Nevertheless, the rate of transport should be more precisely measured in smaller nerve pieces;

this could provide a new approach to studying the turnover of receptors, as already reported for noradrenergic vesicles^{13,14}.

How these prejunctional receptors are transported along the axon remains unclear. However, the following sequence of events can be considered: synthesis of receptors like the proteins¹⁵ and their association with subcellular organelles¹¹ in the perikarya; anterograde flow in the axon; at the terminals externalization of receptors by exocytosis, which makes the receptor available to ligand and thus functional; internalization by endocytosis (see ref. 16), back to perikarya by retrograde axonal flow and recycling or degradation¹⁷. If this is the correct process, most of the binding sites in the perikarya, in the axon and partly in the nerve ending would be silent receptors. If such a view is transposed to the central nervous system, this could provide a plausible explanation for the very limited increase in binding sites in supersensitivity phenomena¹⁸ when compared with the more pronounced pharmacological reactivity.

This work was partially supported by a grant of IWONL. I thank Dr J. Leysen for helpful discussions, D. Ashton for his help in preparing the manuscript, and J. D'Aubioul, W. Van Gerven, W. Gommeren and P. F. M. Janssen for technical assistance.

Received 12 December 1979; accepted 28 April 1980.

1. Dahlström, A. *Phil. Trans. R. Soc. B* **261**, 325–358 (1971).
2. Banks, P. & Mayor, D. *Biochem. Soc. Symp.* **36**, 133–149 (1973).
3. Laduron, P. in *Dynamics of Degeneration and Growth in Neurons* (eds Fuxe, K., Olson, L. & Zetterman, Y.) 245–256 (Pergamon, Oxford, 1974).
4. Lubinska, L. & Niemierko, S. *Brain Res.* **27**, 328–352 (1971).
5. Ochs, S. *J. Physiol. Lond.* **277**, 627–645 (1972).
6. Giachetti, A. & Said, S. I. *Nature* **281**, 574–575 (1979).
7. Langer, S. Z. *Biochem. Pharmacol.* **23**, 1793–1800 (1974).
8. Löffelholz, K. & Muscholl, E. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmacol.* **265**, 1–15 (1969).
9. Vanhoutte, P. M. *Fedn Proc.* **36**, 2444–2449 (1977).
10. Laduron, P. & Belpaire, F. *Life Sci.* **7**, 1–7 (1968).
11. Teichberg, S. & Holtzman, E. J. *Cell Biol.* **57**, 88–108 (1973).
12. Sharma, V. K. & Banerjee, S. P. *Nature* **272**, 276–278 (1978).
13. De Potter, W. P. & Chubb, I. W. *Biochem. J.* **125**, 375–376 (1971).
14. Brimijoin, S. *J. Neurochem.* **19**, 2183–2193 (1972).
15. Droz, B. *J. Microsc.* **6**, 201–228 (1967).
16. Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. *Nature* **279**, 679–685 (1979).
17. Teichberg, S., Holtzman, E., Crain, S. M. & Peterson, E. R. *J. Cell Biol.* **67**, 215–230 (1975).
18. Schwartz, J. C., Costentin, J., Martres, M. P., Protais, P. & Baudry, M. *Neuropharmacology* **17**, 665–685 (1978).
19. Laduron, P. M., Verwimp, M. & Leysen, J. E. *J. Neurochem.* **32**, 421–427 (1979).
20. Gorissen, H., Aerts, G. & Laduron, P. *FEBS Lett.* **96**, 64–68 (1978).
21. Coyle, J. T. & Henry, D. J. *J. Neurochem.* **21**, 61–67 (1973).

Evidence against presynaptic α -adrenoreceptor modulation of cardiac sympathetic transmission

James A. Angus & Paul I. Korner

Baker Medical Research Institute, Commercial Road, Prahran, Victoria, Australia 3181

In addition to its well known actions on postsynaptic adrenoreceptors, noradrenaline released at the sympathetic nerve endings is now believed to modulate subsequent transmitter release through its actions on presynaptic receptors^{1–7}. Much evidence in support of this hypothesis has been obtained from overflow studies in which labelled noradrenaline and metabolites released into the bathing solution are used as an assay of transmitter release. According to Langer⁴ and Rand, McCulloch and Story⁶, evidence to favour the existence of such a presynaptic mechanism includes the demonstration that α -adrenoreceptor antagonists produce enhanced transmitter overflow in steady-state conditions of sympathetic stimulation. They also suggest that α -adrenoreceptor-mediated potentiation of transmitter release would not be expected in response to a

Table 1 Inhibition of stereospecific ^3H -dextetimide binding

	IC ₅₀ (nM)	
	Dog splenic nerves	Rat striatum*
Dextetimide	2.2	1.4
Atropine	6.3	6.8
Oxotremorine	3,200	6,500
Levetimide	28,000	6,500
Nicotine	>100,000	>100,000

*The values were taken from refs 19 and 20.

single pulse, as a threshold concentration of released noradrenaline is required to activate presynaptic inhibition of further release. In the myocardium both conditions have been obtained in the presence of phenoxybenzamine^{5,6}. Surprisingly, although phentolamine is also a potent α -adrenoreceptor antagonist, the potentiation of transmitter release was less than the increase due to phenoxybenzamine⁶. Studies of transmitter overflow have generally been made with supraphysiological sympathetic stimulation of 5 Hz continuously for 30 s or 4 Hz for 60 s (refs 6, 7). We have used more closely physiological stimulation parameters delivered only during the atrial refractory period. We have now shown that phenoxybenzamine markedly potentiates the chronotropic response to a single pulse and also responses up to maximum stimuli. By contrast, phentolamine and yohimbine were totally without effect even at high concentration. The action of phenoxybenzamine was largely accounted for by its effect on uptake blockade. Taken together, our studies provide evidence against a physiological role of presynaptic α -adrenoreceptors in the heart.

Guinea pig right atria were placed horizontally in Krebs' solution (composition in mM: Na⁺ 144, K⁺ 5.9, Ca²⁺ 2.5, Mg²⁺ 1.2, HCO₃⁻ 25, Cl⁻ 128.7, H₂PO₄⁻ 1.2, SO₄²⁻ 1.2, EDTA 0.04, glucose 11) in a 50-ml organ bath at 37 °C with 1 μ M atropine. Two Teflon-coated stainless steel wires, bared only at the tip, were placed on the surface of the atrium approximately 2 mm apart to record the surface electrogram. Following amplification, this signal was used to trigger a microprocessor-controlled period meter with an accuracy of better than 2 ms in the entire system. The cycle to cycle period (interval) was continuously displayed on a chart recorder. When required, intramural sympathetic nerve fibres were stimulated by applying square wave field pulses (2 ms duration, 100 mA) across the atrium from a pair of platinum electrodes. Field pulses were delivered from a Grass S88 stimulator following a 5-ms delay after the upstroke of the atrial electrogram signal (during the refractory period) to prevent arrhythmia⁸. Either a single field pulse or a train of four field pulses (one pulse following each of four consecutive atrial electrograms) were used as stimuli.

In control conditions, these low stimulation frequencies produced a marked tachycardia, that is, a decrease in the succeeding atrial period as measured from the P-P intervals of the atrial electrogram. After a single field pulse applied during the refractory period, atrial period fell by 30 ms, with the fall

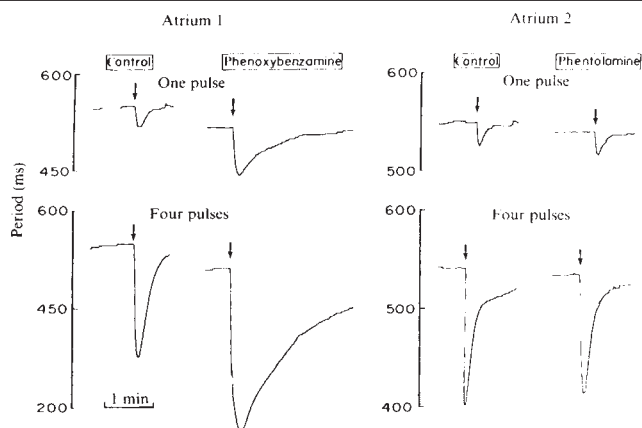


Fig. 1 Chart recordings of right atrial period from two tissues. Top: responses to sympathetic nerve stimulation effected by one square wave field pulse (2 ms duration, 100 mA) delivered during the refractory period in the presence of 1 μ M atropine. Bottom: period responses to four field pulses delivered as a train of one pulse immediately after each of four spontaneous electrogram signals. Phenoxybenzamine (10 μ M) was equilibrated with atrium 1 for 15 min followed by repeated washing for 30 min before restimulation. Phentolamine (10 μ M) was left in contact with atrium 2 for 30 min before, and during, field stimulation. Both the peak fall in period and the duration of the tachycardia were significantly prolonged by phenoxybenzamine treatment but not by phentolamine.

beginning at the next interval. The tachycardia then declined over the next 20 s. The mechanism underlying the time course of response is assumed to depend on the concentration of released noradrenaline in the synaptic cleft. The transmitter will activate postsynaptic β -adrenoreceptors to increase both rate and force of contraction until the concentration falls below the threshold level necessary to activate these receptors. The mechanisms responsible for the lowering of the biophase noradrenaline are a 'neuronal uptake mechanism' (or uptake₁) and an extraneuronal mechanism (uptake₂)^{9,10}. After four pulses the fall in atrial period was approximately 150 ms. Maximum fall in atrial period was 200–250 ms observed with

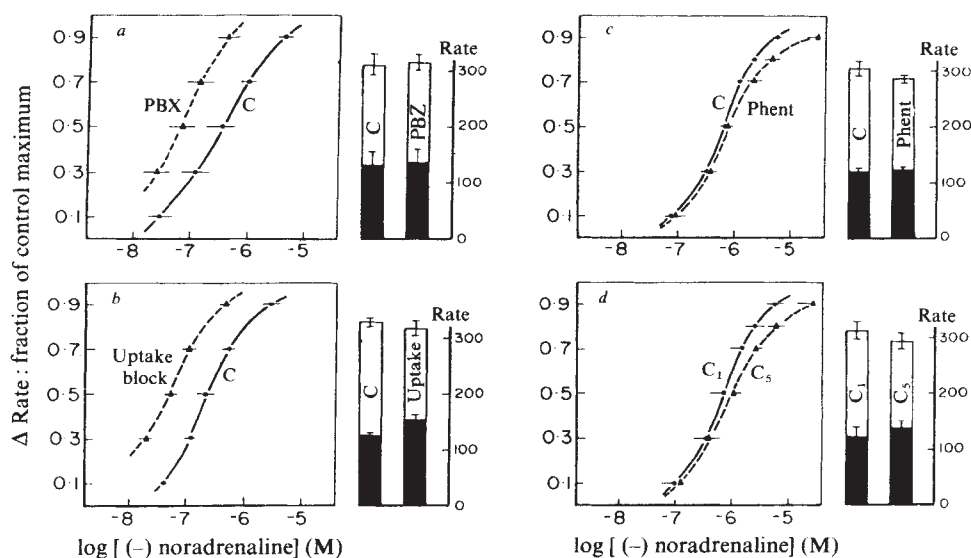
Table 1 Effects of phenoxybenzamine, phentolamine, yohimbine and uptake block on the tachycardia response to sympathetic stimulation in guinea pig atria

Treatment	One pulse				Four pulses							
	Peak (ms)		Area (log units)		Peak (ms)		Area (log units)					
	Before	After	s.e.d.	P	Before	After	s.e.d.	P	Before	After	s.e.d.	P
Control (n=8)	31	31	1.5	NS	0.79	0.84	0.08	NS	134	149	7.8	NS
Phenoxybenzamine												
3 μ M (n=5)	18	39	9.4	NS*	0.78	1.15	0.11	<0.05	113	163	11.6	<0.02
10 μ M (n=10)	41	82	9.6	<0.001	0.83	1.52	0.04	<0.001	184	231	13.9	<0.01
Phentolamine												
0.3 μ M (n=5)	37	31	2.1	<0.05	0.70	0.62	0.04	NS	119	118	3.7	NS
1 μ M (n=5)	30	27	3.2	NS	0.88	0.77	0.07	NS	153	152	19.1	NS
10 μ M (n=12)	29	34	4.6	NS	0.79	0.88	0.06	NS	147	154	10.4	NS
Yohimbine												
0.1 μ M (n=5)	38	40	4.2	NS	0.85	0.91	0.09	NS	138	147	5.3	NS
1 μ M (n=5)	38	41	8.0	NS	0.85	0.83	0.08	NS	138	155	9.3	NS
10 μ M (n=5)	38	39	13.5	NS	0.85	0.87	0.23	NS	138	148	24.6	NS
Uptake block (n=10)	29	54	4.8	<0.001	0.86	1.51	0.07	<0.001	139	176	9.4	<0.005

Peak responses are the maximum fall in period (ms) following either a single pulse or a train of four pulses of square wave field stimulation (2 ms duration, 100 mA) delivered across the atrium by two platinum electrodes during the refractory period of spontaneous sino-atrial rhythm. Area responses are the integrated change in atrial period over the whole response, that is, until the atrial period returns to resting values. The chart record of period was integrated by weighing. Logarithmic units of area were used to obtain homoscedastic observations. Mean values for peak and area responses before and after drug treatment are given together with s.e.d. within atria for each comparison. P values are the level of significance (n is the number of atria in each treatment). Phenoxybenzamine was left in contact with the atria for 15 min followed by repeated washing for 30 min before further field stimulation. Phentolamine, yohimbine and uptake block (combination of desipramine 0.1 μ M and 17 β -oestradiol 5 μ M) were equilibrated with the atria for 30 min and left in the bath during stimulation.

*Considering log (peak) to avoid heteroscedasticity the difference was significant $P < 0.01$.

Fig. 2 Effect of phenoxybenzamine (PBX), phentolamine (Phent.) and uptake block to chronotropic response to exogenous (—)noradrenaline. Graphs: ●, first cumulative concentration-response curve to (—)noradrenaline; ▲, second curve after equilibration of test drug. For each atrium, curves were constructed by first expressing the change in atrial rate as a fraction of the maximum change obtained in the first curve. Mean $\pm$ s.e.m. values of $\log EC_{10}$, EC_{30} , EC_{50} , EC_{70} , EC_{80} and EC_{90} values were determined from four atria. Histograms: black columns, resting atrial rate (beats min^{-1}) just before starting the noradrenaline curve; white columns, maximal atrial rate obtained with noradrenaline. Values are mean $\pm$ s.e.m. for four atria. *a*, Phenoxybenzamine ($10 \mu\text{M}$); 15 min equilibration followed by 30 min washing before second curve. *b*, Uptake block; 30 min equilibration with $0.1 \mu\text{M}$ desipramine and $5 \mu\text{M}$ 17 β -oestradiol before second curve. *c*, Phentolamine ($10 \mu\text{M}$), 30 min equilibration before curve. This curve was the fifth curve on each atrium; the previous curves were in the presence of 0, 0.3, 1 and $3 \mu\text{M}$ phentolamine and were similarly without significant effect on the noradrenaline responses. *d*, Control curves. C_1 refers to the first control curve and C_5 was the fifth curve to noradrenaline in each of four atria. Curves 2, 3 and 4 all lay between these two curves.



8–16 pulses. These findings indicate that the present method of atrial stimulation leads to immediate release of substantial amounts of sympathetic transmitter. Evidence that this transmitter was released from nerves was obtained by the observations that $0.1 \mu\text{M}$ tetrodotoxin completely blocked the response to one and two field pulses and almost abolished the response to four pulses without significantly altering resting atrial rate.

Pretreatment with phenoxybenzamine resulted in a marked potentiation in relation to the control response in both the peak and its duration after either one or four pulses (Table 1 and Fig. 1). If we assume that the tachycardia response is directly related to the synaptic cleft concentration of noradrenaline, the area under the atrial period response record will be a function of the concentration and duration of noradrenaline released by nerve stimulation. The peak responses to a single pulse doubled after phenoxybenzamine whereas the area increased by 2.4- and 4.9-fold after 3 and $10 \mu\text{M}$, respectively (Table 1). In contrast, phentolamine, in concentrations that significantly antagonized α -adrenoreceptors in guinea pig aortic strips, did not increase the tachycardia response with respect to peak or duration to either one or four pulses, nor was there a significant depression of resting atrial period (Table 1 and Fig. 1). Yohimbine, which has been classified as being a more selective presynaptic α -adrenoreceptor antagonist than phentolamine¹¹, was also without significant effect on the response to sympathetic stimulation (Table 1).

Applying four field pulses within one refractory period (that is, four pulses over 40 ms equivalent to a frequency of sympathetic stimulation of $\sim 100 \text{ Hz}$) decreased the atrial period by the same magnitude as four pulses delivered as one pulse following each atrial beat for four beats. However, phentolamine and yohimbine failed to potentiate the responses to either stimulation pattern. These findings contrast with some reports of an increase in the tachycardia response to sympathetic stimulation after phentolamine and yohimbine^{12–15}.

To explore the possibility that phenoxybenzamine potentiated the response to a single pulse by its well established property of uptake inhibition^{9,16}, we investigated the effect of a combination of desipramine and oestradiol, drugs classified as potent inhibitors of uptake₁ and uptake₂ (refs 17, 18). In the guinea pig heart the uptake₂ mechanism is

of minor significance¹⁹ and this was confirmed in the present study by the finding that desipramine alone potentiated the responses to field stimulation as much as did desipramine combined with oestradiol. The potentiation of response to a single pulse in the presence of the combination of the uptake blocking drugs was very similar to the potentiation observed following phenoxybenzamine (Table 1). Moreover, tachycardia responses to cumulative dose-response curves to exogenous noradrenaline were potentiated to a similar degree by phenoxybenzamine and uptake blockade (Fig. 2). By contrast, phentolamine at 0.3 – $10 \mu\text{M}$ had no significant effect on the resting atrial rate or the dose-response curves to noradrenaline (Fig. 2). Therefore, we conclude that uptake

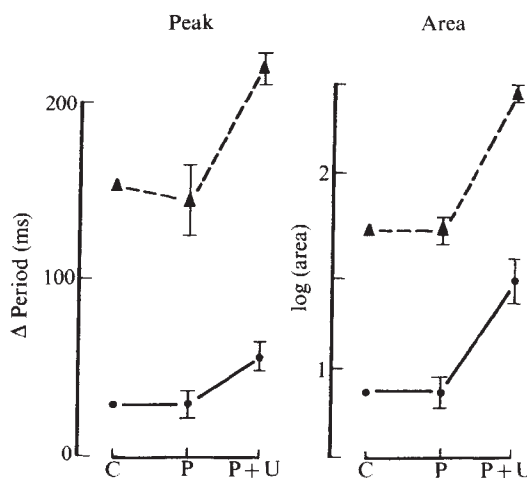


Fig. 3 Potentiation of right atrial period responses to sympathetic stimulation by uptake block in the presence of phentolamine. For each atrium responses were measured to one (●) and four (▲) field pulses in three conditions: (1) C, control, (2) P, in the presence of $10 \mu\text{M}$ phentolamine, and (3) P+U, in the presence of $10 \mu\text{M}$ phentolamine and uptake block ($0.1 \mu\text{M}$ desipramine and $5 \mu\text{M}$ 17 β -oestradiol). Points represent mean values from five atria; bars indicate $\pm$ s.e.d. within atria between adjoining means. All values during P+U were significantly higher than either P or C ($P < 0.05$). Peak and area defined in Table 1 legend.

blockade is more likely to be the underlying mechanism of the phenoxybenzamine potentiation than α -adrenoreceptor antagonism.

The failure of phentolamine to enhance the chronotropic effect of sympathetic nerve stimulation despite a considerable increase in transmitter overflow has been previously observed^{7,20}. However, Langer, Adler-Graschinsky and Giorgi⁷ suggested that phentolamine had a veratramine-like direct depressant action on the sino-atrial node which (1) lowered the resting atrial rate, (2) attenuated the chronotropic response to exogenous noradrenaline and (3) prevented the tachycardia to sympathetic nerve stimulation. We can find no evidence for this depressant action of phentolamine on the resting atrial rate or on exogenous noradrenaline dose-response curves. Moreover, in the presence of a high phentolamine concentration (10 μ M), uptake block treatment potentiated the response to one and four pulses of sympathetic stimulation to a similar degree to uptake block alone (Fig. 3). Similarly, pretreatment with 10 μ M phenoxybenzamine in the presence of 10 μ M phentolamine, still potentiated responses as

before (area responses to one field pulse, log units: phentolamine 0.88; phenoxybenzamine 1.46; s.e.d.=0.097, $t=5.88$, $P<0.01$, seven experiments).

In conclusion, our results indicate that phenoxybenzamine potentiates not only the response to a train of sympathetic nerve pulses but also markedly accentuates the effects of a single pulse. By contrast, the α -adrenoreceptor antagonists phentolamine and yohimbine did not potentiate the effects of either stimulus trains or single pulses. It seems likely that the potentiation produced by phenoxybenzamine was not due to its α -adrenoreceptor antagonist action but to its property of uptake block. This suggests that presynaptic α -adrenoreceptor modulation by synaptically released noradrenaline plays no part in cardiac sympathetic transmission, at any rate, at the physiological stimulus levels of the present experiments which were generally lower than those used in overflow studies.

We thank Mrs E. Anderson and Mr M. Lew for technical assistance and Mr M. Percy for the period meter. J.A.A. is a holder of an NH and MRC of Australia C.J. Martin Fellowship.

Received 30 November 1979; accepted 16 April 1980.

1. Farnebo, L. O. & Hamberger, B. *Br. J. Pharmac.* **43**, 97-106 (1971).
2. Enero, M. A., Langer, S. Z., Rothlin, R. P. & Stefano, F. J. E. *Br. J. Pharmac.* **44**, 672-688 (1972).
3. Starke, K. *Naunyn-Schmiedeberg's Archs Pharmac.* **275**, 11-23 (1972).
4. Langer, S. Z. *Br. J. Pharmac.* **60**, 481-497 (1977).
5. Rand, M. J., Story, D. F., Allen, G. S., Glover, B. & McCulloch, M. W. in *Frontiers in Catecholamine Research* (eds Usdin, E. & Synder, S. H.) 579-581 (Pergamon, Oxford, 1973).
6. Rand, M. J., McCulloch, M. W. & Story, D. F. in *Central Action of Drugs in Blood Pressure Regulation* (eds Davies, D. S. & Reid, J. L.) 94-132 (Pitman, Tunbridge Wells, 1975).
7. Langer, S. Z., Adler-Graschinsky, E. & Giorgi, O. *Nature* **265**, 648-650 (1977).
8. Blinks, J. R. *J. Pharmac. exp. Ther.* **151**, 221-235 (1966).
9. Paton, D. M. in *The Mechanism of Neuronal and Extraneuronal Transport of Catecholamines* (ed. Paton, D. M.) 49-66 (Raven, New York, 1976).
10. Iversen, L. L. *The Uptake and Storage of Noradrenaline in Sympathetic Nerves* (Cambridge University Press, 1967).
11. Starke, K., Borowski, E. & Endo, T. *Eur. J. Pharmac.* **34**, 385-388 (1975).
12. Lockhandwala, M. F. & Buckley, J. P. *Eur. J. Pharmac.* **40**, 183-186 (1976).
13. Caverio, I. et al. *Br. J. Pharmac.* **67**, 283-292 (1979).
14. Mouill , P., Huchet, A.-M., Lucet, B., Chelly, J. & Schmitt, H. *J. cardiovascular Pharmac.* **1**, 515-528 (1979).
15. Docherty, J. P. & McGrath, J. C. *Br. J. Pharmac.* **66**, 55-63 (1979).
16. Iversen, L. L. *Adv. Drug. Res.* **2**, 5-23 (1965).
17. Story, D. F., Story, M. E., McCulloch, M. W., Hope, W. & Rand, M. J. *Clin. exp. Physiol. Pharmac.* **1**, 429-439 (1974).
18. Salt, P. J. *Eur. J. Pharmac.* **20**, 329-340 (1972).
19. Trendelenburg, U. *Trends pharmac. Sci.* **1**, 4-6 (1979).
20. Adler-Graschinsky, E. & Langer, S. Z. *Br. J. Pharmac.* **53**, 43-50 (1975).

Open time of channels activated by binding of two distinct agonists

Alain Trautmann & Anne Feltz*

Laboratoire de Neurobiologie, Ecole Normale Sup rieure 75230 Paris Cedex 05, France

At the frog endplate, acetylcholine (ACh) causes depolarization by opening synaptic channels, and it is generally assumed that more than one molecule must bind to open one channel. This interpretation, proposed to explain the sigmoidicity of the dose-response curve^{1,2}, also accounts for the potentiating effect of pipette-released ACh on the response to nerve-released ACh³. We have described a similar cross-potentiation between ACh and carbachol (CCh)^{4,5}. The response to simultaneous application of these two agonists is larger than the sum of the responses to separate application, and we have assumed that this reflects the binding of both ACh and carbachol to individual receptor molecules. One approach to an understanding of the molecular basis of transmitter action is to study the mean time a channel remains open in its presence. Because this mean 'open time' depends on the nature of the agonist used to activate a given receptor, we have measured this parameter in order to understand better the mechanism underlying the cross-potentiation. We compared the 'open times' observed when the two agonists were applied separately with those obtained when both were applied together. In each case, when a 'faster' agonist (one inducing 'short' open times) and a 'slower' agonist (one inducing 'long' open times) are applied together, the channels opened by combined activation have a short mean open time, as if these were only controlled by the faster agonist.

The open time of a channel at the frog endplate (τ) can be measured directly (by single-channel recording⁸), or it can be estimated indirectly from the relaxation of the endplate current when displaced from equilibrium. Displacement can be spontaneous, appearing in the form of fluctuations of thermal origin around a mean, and in that case noise analysis is used⁶; it can be caused by a concentration jump, in which case the decay of the endplate current is measured⁷; or, as τ is voltage-dependent, it can be caused by voltage jumps⁹⁻¹¹. The estimation of τ is effectively independent of the method used, at least at a low concentration of agonist. We used the last method, where τ is given by the time constant of relaxation of the agonist-induced current after an instantaneous voltage jump.

Experiments were carried out on the frog endplate at a low temperature (3-5°C) to reduce the rate of desensitization^{5,12}. Membrane potential was controlled by a conventional two-electrode voltage clamp on an endplate region located under Nomarski optics. Identical voltage jumps were performed before (control) and after application of various agonists. Pure agonist-induced current was obtained by subtracting from the current measured in the presence of the agonist (before, during and after the voltage step) the 'nonsynaptic' current measured during a control jump⁹⁻¹¹. Figure 1a (top trace) shows the CCh-induced current obtained by such a subtraction. Before the jump, at -90 mV, the agonist induced a current of 20 nA. After hyperpolarization to -140 mV, the CCh-induced current first increased as expected from the change in driving force on the channels that were open before the jump. The current then relaxed towards a new steady level. When the membrane potential was returned to the initial holding potential, the CCh-induced current first decreased rapidly, then more slowly, eventually returning to the value measured before the voltage step.

A semilogarithmic plot of current against time (Fig. 1b) shows that after reaching the new voltage (-140 mV), the

*Present address: Laboratoire de Physiologie Respiratoire, CNRS, 23 rue Beccuercel, 67087 Strasbourg, France.

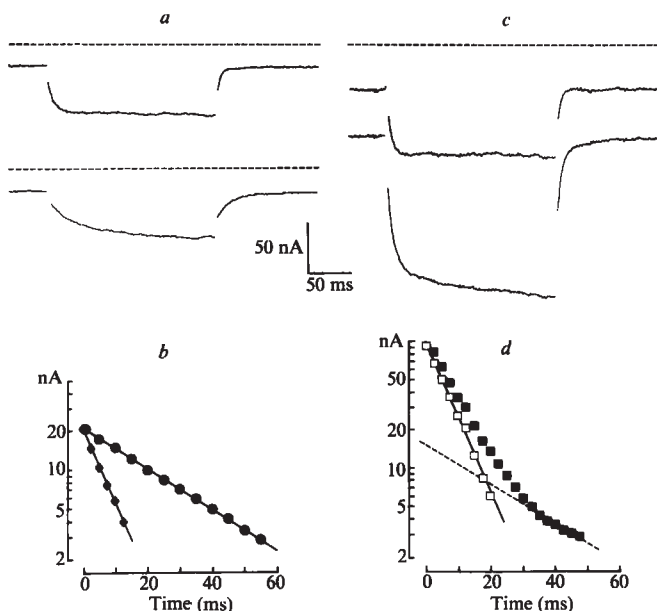


Fig. 1 Agonist-induced currents during a voltage jump lasting 205 ms from a -90 -mV to a -140 -mV holding potential. The voltage jump was performed before, then during agonist application. *a*, Current induced by the application of $10\text{ }\mu\text{M}$ CCh (top trace) and 37 nM SubCh (bottom trace). *b*, Semilog plot of the CCh-induced current (rhombi) and SubCh-induced current (circles) against time, after a jump at -140 mV. Both currents decay exponentially, with time constants of 7.6 ms and 27 ms respectively. *c*, The bottom trace shows the 'total current' resulting from the simultaneous application of 37 nM SubCh and $10\text{ }\mu\text{M}$ CCh, and the top trace shows the difference between the total current and the sum of the two currents given in *a*. *d*, Semilog plot of the total current (black squares) against time after a jump at -140 mV; the foot of the curve is fitted with an exponential (dotted line) of time constant 27 ms as in *b*. The difference between the total current and this slow component is given by the open squares, which can be fitted by an exponential with time constant 7.5 ms. The time constant of the 'difference' shown in *b*, top (semilog plot is not shown) is 5.7 ms. This figure is somehow shorter than the fast component, but this difference was not systematic in the various experiments (see text). The base line slopes slightly on all records; its time constants of several hundreds of ms are easily separable from the values we are studying. The perfusing medium was cooled at 3°C to reduce desensitization. In the same line, a Ca-free Ringer was used, with the following composition (mM): NaCl, 115; KCl, 2; MgCl_2 , 5; HEPES 2 mM and pH adjusted to 7.2. All data were recorded on a tape recorder Racal M4 and later analysed on a Hewlett Packard 9825A desk computer. Note the increase in noise level in *c* (compared with *a*): the phenomenon was recorded at a low gain (to avoid saturation on the tape), and reamplified later, with a resulting increase of the background noise from the tape.

current relaxed exponentially to a steady level, enabling us to estimate, at this potential, the mean open time of the channels controlled by CCh (τ_{CCh}).

Similar records obtained during the application of suberyldicholine (SubCh) show slower relaxations (Fig. 1*a*, bottom trace). In the voltage range studied, τ_{CCh} and τ_{SubCh} differ by a factor of 3–4, in good agreement with previous data^{6,11}. Both values increase with hyperpolarization, and both double over a 50 -mV range.

At the holding potential of -90 mV, separate applications of $10\text{ }\mu\text{M}$ CCh and 37 nM SubCh resulted in an inward current of 20 nA and 25 nA, respectively. During simultaneous application of both agonists ($10\text{ }\mu\text{M}$ CCh + 37 nM SubCh) cross-potentiation was observed: instead of the 45 -nA current

expected from purely additive effects, a total current of 87 nA developed. Moreover, when SubCh and CCh were applied together, the relaxation following a voltage jump was no longer exponential, but biphasic. The extra current produced by cross-potentiation was calculated by subtracting both the CCh-induced current and the SubCh-induced current shown in Fig. 1*a* from the agonist-induced current which developed during simultaneous application. Figure 1*c* (top trace) shows that this extra current relaxed along a single exponential with a time constant almost identical to that observed with CCh alone.

The subtraction of the two single-agonist relaxations from the double-agonist relaxation is justifiable only if the number of receptors bound by two molecules of a given agonist is the same when that agonist is applied alone and when it is applied in combination with the second agonist of the pair—a criterion that can be assumed to be met if the fraction of receptors activated by the agonist is small. In our experimental conditions, clearly only a small proportion of the receptors were activated, because the doses used induced a current of only 20 nA ($10\text{ }\mu\text{M}$ CCh), whereas the current expected from activation of all the receptors (assuming a total of more than 10^7 receptors per endplate¹³ and an elementary conductance of 25 pS^{14}) is $1,000$ times greater. Furthermore, because low concentrations of agonist and low temperatures were used, desensitization developed very slowly.

Given our experimental conditions, it thus seems possible to conclude that the receptor population was far from saturated and that desensitization was avoided, so that we could assume that the additional current seen with combined agonist application was due to the recruitment of a new group of receptors, each bound by one CCh and one SubCh molecule. Even if receptor saturation and desensitization were not completely avoided, neither of these factors would be expected to alter our principal finding—that the extra channels controlled by combined agonist application are opened for a short time.

The same conclusion as that drawn from subtracting the two relaxations can be reached directly from a semilog plot of the relaxation of the combined agonist-induced current against time (Fig. 1*d*). Two exponentials appear, with time constants the same as those obtained by separate application of the two agonists. Whereas the amplitude of the slow component remained equal to that obtained with SubCh alone, that of the fast component was four times larger than in the relaxation seen with CCh alone. This again suggests that the channels opened by combined application of SubCh and CCh behave as CCh-activated channels.

To investigate whether our observations were generally applicable, we looked at the characteristics of the channels during cross-potentiation between ACh and acetylthiocholine, SubCh, or CCh. These experiments were all performed after irreversible inactivation of acetylcholinesterase by *O*-ethyl- S_2 -diisopropyl-amino-ethyl-methyl-phosphonothionate (MTP)⁵. In all cases, the agonist, which alone would have led to the shorter open time, τ_{short} (so called faster agonist), seemed to determine the open time of the channels controlled by two agonists (τ_{pot}). In 12 experiments $\tau_{\text{pot}}/\tau_{\text{short}}$ was 1.08 ± 0.31 ($\pm$ s.d.).

Our data thus suggest that the binding of a receptor molecule by two different agonists leads to channel opening for a time determined in most cases, if not always, by the faster of the two agonists. These results have to be taken into account in schemes describing normal channel activation. It has often been suggested that sequential binding of two agonist molecules is followed by isomerization of the receptor-channel complex and subsequent channel opening (see ref. 14 for a review). Our data are not easily interpreted with respect to such a model: if isomerization is the rate limiting step, it has to be assumed that for a mixed activation, isomerization depends only on the faster agonist, and that is not very satisfying. On the other hand, if binding is the rate limiting

step, one can predict that the open time of the channels controlled by receptors bound by two agonists will be intermediate between the values characteristic of each agonist, which we did not find. A more general discussion would have to take into account other possibilities—if the two binding sites on one receptor are not identical, then models compatible with our results are possible.

This work was supported by grants from CNRS, INSERM (ATP 58-78-90) and DGRST. We thank P. Ascher and J. Kehoe for comments on the manuscript. Suberyldicholine was given by J. Heisemann and B. Sakmann.

Received 14 February; accepted 28 April 1980.

1. Katz, B. & Thesleff, S. *J. Physiol., Lond.* **138**, 63–80 (1957).
2. Jenkinson, D. H. *J. Physiol., Lond.* **152**, 309–324 (1960).
3. Hartzell, H. C., Kuffler, S. W. & Yoshikami, D. *J. Physiol., Lond.* **251**, 427–463 (1975).
4. Feltz, A. & Trautmann, A. *J. Physiol., Lond.* **293**, 64–65P (1979).
5. Feltz, A. & Trautmann, A. *J. Physiol., Lond.* **299**, 533–552 (1980).
6. Katz, B. & Miledi, R. *J. Physiol., Lond.* **230**, 707–717 (1973).
7. Magleby, K. L. & Stevens, C. F. *J. Physiol., Lond.* **223**, 151–171 (1972).
8. Neher, E. & Sakmann, B. *Nature* **260**, 799–801 (1976).
9. Adams, P. R. *Br. J. Pharmac.* **53**, 308–310 (1975).
10. Sheridan, R. E. & Lester, H. A. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3496–3500 (1975).
11. Neher, E. & Sakmann, B. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2140–2142 (1975).
12. Magazanik, L. G. & Vyskocil, F. *J. Physiol., Lond.* **249**, 285–300 (1975).
13. Matthews-Bellinger, J. & Salpeter, M. M. *J. Physiol., Lond.* **279**, 197–213 (1978).
14. Colquhoun, D. in *The Receptors* 1 (ed. O'Brien, R. D.) 93–142 (Plenum, New York, 1979).

Proton NMR detection of acetylcholine status in synaptic vesicles

H. Stadler & H. H. Földner

Max-Planck-Institut für biophysikalische Chemie, Postfach 968, D 3400 Göttingen 1, FRG

The storage granules of cholinergic nerve terminals, the synaptic vesicles, have a central role in the mechanism of cholinergic transmission, and direct evidence as to whether acetylcholine is stored in them in a free or partially immobilized form is therefore of interest. In some secretory systems it has become evident that low molecular weight secretion products are stored within the granules as complexes with proteins or proteoglycans¹. The application of high resolution NMR spectroscopy to isolated granules^{2–4} has recently elucidated the internal structure of secretory granules. We report here that proton NMR analysis of isolated cholinergic synaptic vesicles from *Torpedo marmorata* allows the identification of acetylcholine within the organelle. The spectrum shows that all the acetylcholine is dissolved in an essentially fluid phase within the core. Vesicular and uncompartimentalized acetylcholine can be distinguished from each other and in addition, any hydrolysis to choline and acetate can be monitored. The results open the possibility of studying dynamics of acetylcholine pools and their breakdown in cholinergic tissue by a direct and non-perturbing method.

After isolation⁵, vesicles were pelleted, resuspended in a small volume, dialysed against isosmolar D₂O buffer and spectra recorded in a Bruker WH 270 FT spectrometer interfaced to a Nicolet B-NC 12 computer. Chemical shifts are given relative to the internal standard 3-(trimethylsilyl)-d₄-propionic acid, whose pH dependence has been observed where necessary⁶. Complete recording of integrals was assured by proper choice of the acquisition parameters. The temperature was kept at 5 °C.

Basically, three experiments were carried out. In the first experiment, the spectrum of intact vesicles was recorded (Fig. 1a). In the second, the acetylcholinesterase present as a contaminant⁵ in the sample was blocked by diethyl-*p*-nitrophenyl phosphate (paraoxon). The vesicles were then lysed by several freeze-thaw cycles and the spectrum was taken (Fig.

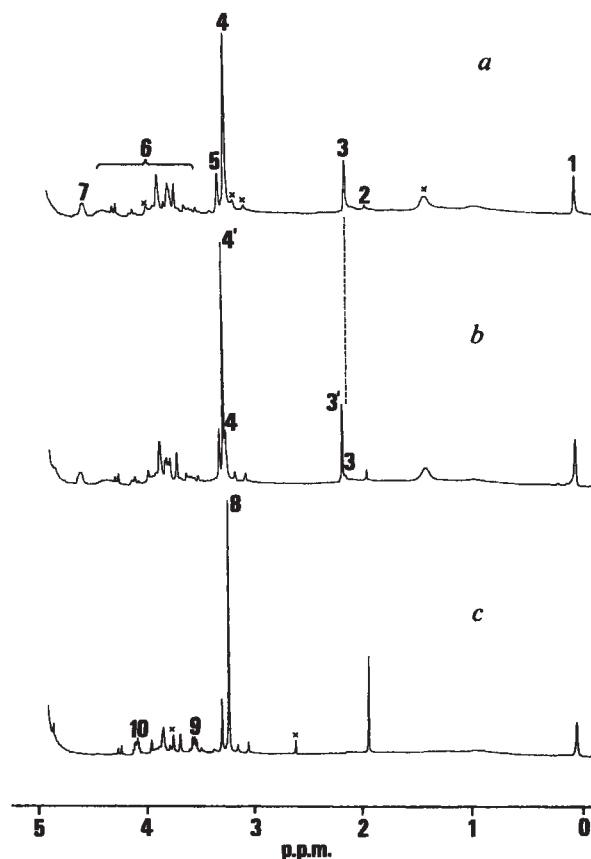


Fig. 1 Proton NMR spectra of isolated cholinergic synaptic vesicles from *Torpedo marmorata*. Vesicles were pelleted from the corresponding fraction from a sucrose density step gradient and suspended and dialysed against 0.4 M NaCl and 20 mM phosphate pD 7.4 made in D₂O and the spectrum recorded at 5 °C. a, Intact vesicles in the presence of 50 µM paraoxon (acetylcholine esterase blocker). b, The same preparation as a, but after lysis of vesicles and release of acetylcholine by repeated freezing and thawing. The dashed line indicates the chemical shift between resonances 3 and 3', the shift is present on all acetylcholine resonances (see text). c, Another vesicle preparation that has been lysed by freezing and thawing in the absence of an acetylcholine esterase blocker. The acetylcholine has been hydrolysed by the esterase present in the sample. Identified resonances: (1) 3-(trimethylsilyl)-propionic acid (internal standard, 1 mM); (2) free acetate; (3) and (3') -CH₃ protons of the acetyl residue of intact acetylcholine; (4) and (4') -N-(CH₃)₃ protons of acetylcholine; (5) trimethyloxamine; (6) resonances from residual sucrose and -CH₂- protons from acetylcholine; (7) -CH₂- protons of acetylcholine adjacent to the acetyl group; (8) -N-(CH₃)₃ protons of choline; (9) and (10) -CH₂- protons of choline superimposed on sugar resonances; (x) unidentified resonances.

1b). In the third experiment the esterase was left active, the vesicles were lysed and a spectrum with the resulting choline and acetate resonances was obtained (Fig. 1c).

The simple spectrum of intact vesicles (Fig. 1a) contains the resonances of acetylcholine, trimethyloxamine (present in high concentrations in the body fluids of elasmobranchs⁷) and residual sucrose, together with a few minor unidentified resonances. Although ATP is present in vesicles in a 1:7 ratio relative to acetylcholine⁵, its resonances could not be identified unambiguously. The membrane lipids gave only a very broad background.

Figure 2 shows the changes in the spectral region containing the N(CH₃)₃ and acetyl-CH₃ resonances when the vesicle preparation was subjected to several freeze-thaw cycles in the presence of the anticholinesterase. The progressive release of the intact acetylcholine as vesicles are broken is apparent.

Three observations were noted: (1) The acetylcholine resonances of intact vesicles are shifted upfield relative to free acetylcholine as obtained after lysis or from a simple acetylcholine solution in our buffer (spectrum not shown). The shift ranges from about 0.02 p.p.m. to 0.04 p.p.m. for the various acetylcholine resonances (compare Fig. 1a, b). Although small, it is reproducibly above the resolution limit of 0.01 p.p.m. Possible explanations for this shift include susceptibility differences between the vesicle core and the external medium, differences in pH or ionic strength, and a weak interaction of acetylcholine with the negatively charged groups of a glycosaminoglycan found to be associated with the vesicles⁸. The effect of pH and ionic strength was insufficient to explain the observed shifts. (2) The integrals of corresponding resonances are identical before and after lysis. This means that all the vesicular acetylcholine is detected in the intact vesicles. (3) The stored form of acetylcholine shows only slightly bigger linewidths than the released one. Therefore, there is no doubt that the stored acetylcholine is dissolved; the differences in linewidths are presumably due to viscosity differences.

The result of the third experiment (several freeze-thaw cycles in the presence of active cholinesterase) is presented in Fig. 3. Here, vesicle breakdown and the immediate hydrolysis of the released acetylcholine are indicated by the disappearance of the acetyl-CH₃-resonance of intact acetylcholine and the concomitant appearance of the CH₃-resonance of free acetate. Correspondingly, all other acetylcholine resonances change over to those of choline (see Fig. 1c). This observation supports our deduction from the chemical shifts in the spectrum of acetylcholine that these were due to the difference between intra- and extravesicular acetylcholine. Another experiment of type 3 (omitting the freeze-thaw cycles) established that the half life of

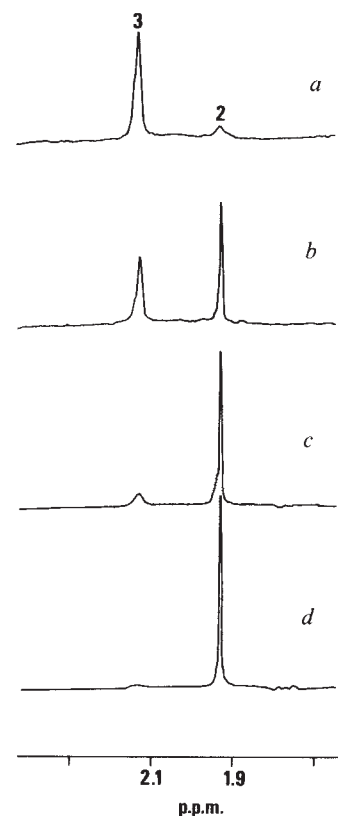


Fig. 3 Hydrolysis of acetylcholine progressively released from synaptic vesicles in the presence of unblocked acetylcholine esterase. Conditions as described in Fig. 1c, labelling as in Fig. 1. Part of the spectrum shown in Fig. 1c is presented. a-d Are spectra after 0, 2, 8 and 16 freeze-thaw cycles, respectively. Freezing and thawing of an acetylcholine solution in the same condition has no effect.

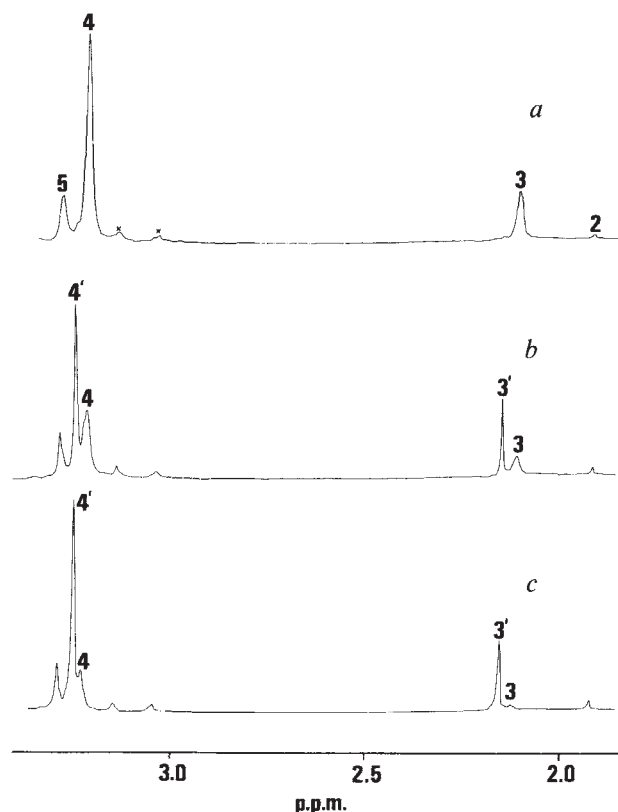


Fig. 2 Proton NMR spectra of cholinergic synaptic vesicles as a function of progressive release of acetylcholine from the vesicles by repeating freezing and thawing. Conditions and labelling as in Fig. 1a, b; a window of the resonances (2) to (5) is presented on an expanded scale. a, Intact vesicles before freezing and thawing; b, after 4 freeze-thaw cycles; c, after 12 freeze-thaw cycles.

acetylcholine (~36 h) is long compared with the time of the experiments.

We accordingly conclude that the internal organization of acetylcholine in synaptic vesicles, in agreement with our recent findings that no soluble or easily releasable high molecular weight material is present in the core^{5,13}, constitutes a specialized version of a secretory organelle with fully dissolved storage product, thus fulfilling the need for quick acetylcholine release, which presumably occurs via exocytosis of vesicles during synaptic transmission. The result is consistent with density measurements suggesting that the vesicular acetylcholine is in an 'osmotically active' state^{9,10}.

As there is evidence that the dynamic structure of the internal content of an isolated secretory granule is identical with that of the granule in the intact organ¹¹, the shift differences mentioned might provide the basis for future NMR experiments using synaptosomes or intact (perfused) tissue to study the turnover and breakdown of acetylcholine pools, a matter of current interest¹².

We thank Dr V. P. Whittaker for support and Frl. U. Welscher for technical assistance. The work was supported by the SFB 33 project C6.

Received 6 February; accepted 23 April 1980.

- Giannattasio, G., Zanini, A. & Meldolesi, J. in *Complex Carbohydrates of Nervous Tissue* (eds Margolis, R. U. & Margolis, R. K.) 338 (Plenum, New York, 1979).
- Daniels, A., Korda, A., Fanswell, P., Williams, A. & Williams, R. J. P. *Proc. R. Soc. B* **187**, 353 (1974).
- Casey, R. P., Njus, D., Radda, G. K. & Sehr, P. A. *Biochemistry* **16**, 972 (1977).
- Ugurbil, K., Holmsen, H. & Shulman, R. G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2227 (1979).
- Tashiro, T. & Stadler, H. *Eur. J. Biochem.* **90**, 479 (1978).
- De Marco, A. *J. magn. Resonance* **20**, 527 (1977).
- Cohen, J. J., Krupp, M. A. & Chidsey, C. A. *Am. J. Physiol.* **194**, 229 (1958).
- Stadler, H. & Whittaker, V. P. *Brain Res.* **153**, 408 (1978).
- Breer, H., Morris, S. J. & Whittaker, V. P. *Eur. J. Biochem.* **81**, 453 (1978).
- Ohsawa, K., Dowe, G. H., Morris, S. J. & Whittaker, V. P. *Brain Res.* **161**, 447 (1979).
- Daniels, A., Williams, R. J. P. & Wright, P. E. *Nature* **261**, 321 (1976).
- Zimmermann, H. *Neuroscience* **5**, 1773 (1979).
- Stadler, H. *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 326 (1978).

Metallothionein mRNA induction in HeLa cells in response to zinc or dexamethasone is a primary induction response

Michael Karin, Robert D. Andersen, Emily Slater, Karen Smith & Harvey R. Herschman

Molecular Biology Institute, Department of Biological Chemistry, and Laboratory of Nuclear Medicine and Radiation Biology, UCLA School of Medicine, Los Angeles, California 90024

Metallothioneins (MTs) are low molecular weight, heavy metal binding proteins unique in their high cysteine content and high affinity for Zn^{2+} , Cd^{2+} , Hg^{2+} , Ag^{2+} and Cu^{2+} (refs 1–3). The synthesis of MTs is induced by zinc or cadmium in the liver and kidney^{4,8} and in cultured cells^{9–12}. More recently MT induction by the steroid hormone dexamethasone (Dex) has been demonstrated in HeLa cells¹³ and adrenalectomized rats¹⁴. Because glucocorticoid hormones lead to an intracellular accumulation of zinc^{15–17}, the question arises of whether the induction of MT gene expression by steroids is a 'primary induction response' (ref. 18), or due to elevated intracellular Zn^{2+} . The glucocorticoid-induced transport of Zn^{2+} is dependent on concurrent protein synthesis^{10,19}. We now show that, in contrast to glucocorticoid-stimulated Zn^{2+} transport, the Zn^{2+} and Dex induction of translatable MT-mRNA is independent of concomitant protein synthesis but not RNA synthesis; that is, MT induction by either agent is a primary induction response¹⁸.

We first compared the effects of Dex and Zn^{2+} on (1) the patterns of protein synthesis directed by total HeLa cytoplasmic RNA in a cell-free system, and (2) the patterns of soluble protein synthesis in the cells (Fig. 1). HeLa cells were grown as previously described¹³. To demonstrate an elevated MT-mRNA level in response to Zn^{2+} or Dex, confluent plates of HeLa cells were incubated for 6 h in control medium, 10^{-7} M Dex, or 3.7×10^{-5} M Zn^{2+} . At the end of this induction period the cells were collected by scraping, with a rubber policeman, into ice-cold phosphate-buffered saline. The cells were collected by centrifugation in a Beckman Microfuge B for 45 s. Total cytoplasmic RNA was extracted from the cells by the phenol-chloroform method of Anderson *et al.*²⁰. Total cytoplasmic RNA extracted from HeLa cells was translated in the wheat-germ cell-free translation system described by Andersen and Weser²¹ for translation of rat liver MT-mRNA. The incorporation of radioactivity into MT is a linear function of the amount of HeLa cell total cytoplasmic RNA added to the translation system up to 15 μg of total RNA per reaction mixture (data not shown). To examine the effects of Zn^{2+} and Dex on MT synthesis in the cells, additional plates were exposed to inducers for 6 h, shifted to cysteine-free medium and exposed for an additional hour to ^{35}S -cysteine (see Fig. 1 legend).

At the end of either the *in vitro* translation reaction or the radioactive labelling of the cultured cells, the fractions to be analysed were reduced and alkylated (ref. 21 and Fig. 1 legend) and subjected to electrophoresis on polyacrylamide gels. Due to its high cysteine content, reduced and carboxymethylated MT has a large net negative charge (carboxymethyl cysteine is negatively charged in the conditions used for electrophoresis). As a consequence, the carboxymethylated MT migrates abnormally rapidly on 20% polyacrylamide SDS gels (see ref. 21). In this gel system MTs migrate as a single band with an R_F of 0.72 relative to bromophenol blue, and are completely separated from all other proteins²¹.

Lanes a, c and e of Fig. 1 are the reduced and carboxymethylated proteins synthesized in the cell-free system.

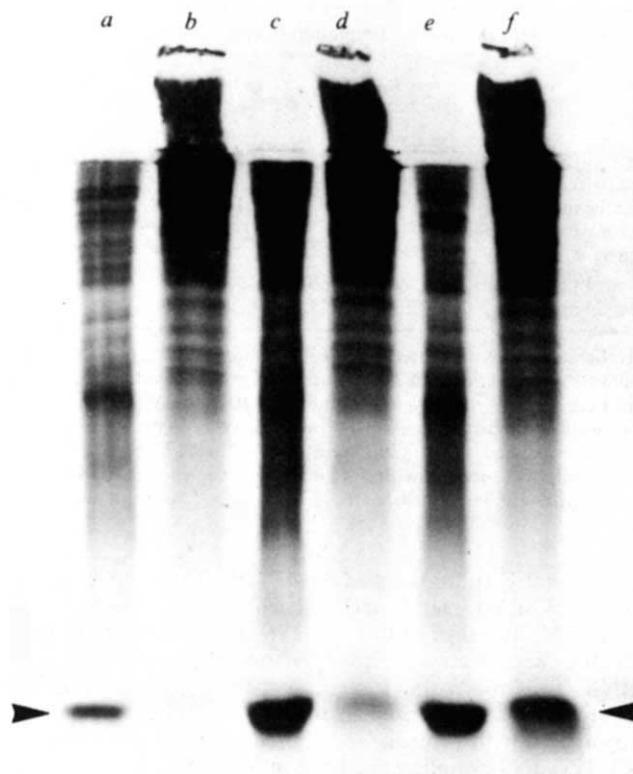


Fig. 1 Dexamethasone- and Zn^{2+} -induced stimulation of MT synthesis and MT-mRNA accumulation in HeLa cells cultured in serum-free medium. HeLa cells were incubated for 6 h with control medium (a, b), medium + 10^{-7} M Dex (c, d), or medium + 3.7×10^{-5} M Zn^{2+} (d, e). At the end of the incubation period three culture plates for each point were collected and total cytoplasmic RNA was extracted. The cell-free translation was carried out as described by Andersen and Weser²¹ in a total volume of 25 μl , optimal salt concentrations were 4.5 mM MgCl_2 and 65 mM KCl. Unlabelled cysteine was omitted from the amino acid mixture and replaced with 0.8 μM of ^{35}S -cysteine (translation grade, NEN 608 Ci mmol^{-1}). The reaction was initiated by adding exogenous RNA. All samples were assayed with both 10 μg (a subsaturating amount) and 15 μg (the peak of the concentration curve) of cellular RNA. At the end of the 2-h incubation at 25 °C the samples were carboxymethylated as previously described²¹. The remaining culture dishes were labelled from the 6th to the 7th hour with 30 μCi of ^{35}S -cysteine (NEN, 300–500 Ci mmol^{-1}). Radioactive labelling was carried out in cysteine-free medium containing the appropriate inducers. At the end of the 1-h pulse, the cells were collected and the soluble proteins extracted as described before¹³. Reduction and alkylation of proteins synthesized in the cells were done as follows. To 200 μl of the cell extract were added 20 μl of 1 M HCl and 20 μl of 0.25 M EDTA. After 20 min at room temperature, 300 μl of 8 M guanidine, 0.5 M Tris-Cl, 0.05 M EDTA, pH 8.5, containing 20 mM dithiothreitol were added. After 3 h incubation at 37 °C, 50 μl of 2 M neutralized iodoacetic acid were added and the mixture was incubated for another 90 min at 37 °C. The reaction was terminated by dialysing against H_2O for 24 h. All the samples were subjected to electrophoresis on 20% polyacrylamide, 0.1% SDS slab gels. Lane a, 76,000 c.p.m. of translation product from the cell-free protein synthesis reaction, synthesis directed by RNA from control cultures. Lane b, 151,000 c.p.m. of intracellular ^{35}S -cysteine-labelled soluble proteins from control cultures. Lane c, 81,000 c.p.m. of *in vitro* synthesized translation product directed by RNA from Dex-induced cultures. Lane d, 161,000 c.p.m. of intracellular labelled soluble proteins from Dex-induced cultures. Lane e, 77,000 c.p.m. of *in vitro* synthesized translation products directed by RNA from Zn^{2+} -induced cultures. Lane f, 162,000 c.p.m. of intracellular labelled soluble proteins from Zn^{2+} -induced cultures. Electrophoresis and fluorography have been described previously^{21,25}. The position of the MT band is indicated by the arrows.

The reduced and carboxymethylated soluble proteins extracted from ^{35}S -cysteine-labelled cells are in lanes b, d and f. The MT band (indicated by the arrows) is markedly increased by Dex (lanes c, d) and by Zn^{2+} (lanes e, f) in comparison with control cells (lanes a, b). Dex and Zn^{2+} clearly stimulate MT synthesis both in the cells and in the RNA-dependent *in vitro* translation system. Characterization, by both DEAE-Sephadex chromatography and electrophoresis on native polyacrylamide gels, of the Zn^{2+} - and Dex-induced MT from HeLa cells

Table 1 Dexamethasone stimulation of zinc uptake by HeLa cells

	Expt 1 (confluent cells)	Expt 2 (confluent cells)	Expt 3 (exponential cells)
Control (6 h)	37,734 ± 953	37,543 ± 3016	21,474 ± 3772
Dex (6 h)	43,899 ± 220	43,140 ± 1689	26,200 ± 2848
Cycloheximide (6 h)	—	31,144 ± 1288	19,040 ± 2206
Dex + cycloheximide (6 h)	—	26,729 ± 3858	18,938 ± 891
Control (24 h)	57,903 ± 2780	—	—
Dex (24 h)	97,647 ± 2475	—	—

HeLa cells were incubated for the times indicated in our serum-free medium¹³ with 0.5 μ Ci of $^{65}\text{Zn}^{2+}$ per plate. Dexamethasone, where indicated, was 10^{-7} M; cycloheximide was 10^{-4} M. At the end of the incubation period the cells were washed twice with 5 ml of cold phosphate-buffered saline. Cells were dissolved in 1 M NaOH. $^{65}\text{Zn}^{2+}$ content was measured in a Beckman Biogamma counter. Expts 1 and 2 were with confluent cells; expt 3 was with cells at half-maximal density.

suggests that both forms of MT (MT_I and MT_{II}, refs 2, 12) are induced by heavy metals and glucocorticoid hormones (M.K. and H.R.H., in preparation). R.A. *et al.*²⁰ have demonstrated that the wheat-germ system preferentially translates small mRNAs *in vitro*. We also see a reduction in high molecular weight proteins synthesized in the cell-free system (lanes a, c, e) compared with proteins synthesized in intact cells (lanes b, d, f). This does not affect the translation of MT-mRNA, which codes for a low molecular weight (6,000) protein.

To determine whether or not the elevation of MT-mRNA in response to the two different inducers is dependent on *de novo* synthesis of RNA and protein, we used inhibitors of RNA and protein synthesis (actinomycin D and cycloheximide). HeLa cells were exposed for 6 h to 10^{-7} M Dex or 3.7×10^{-5} M Zn^{2+} , either in the absence or presence of inhibitors. At the end of the 6-h exposure period total cytoplasmic RNA was extracted and translated in the cell-free system, using ^{35}S -cysteine to label the proteins synthesized. The reduced and carboxymethylated proteins were subjected to electrophoresis and fluorograms were prepared (Fig. 2). Lanes a, b and c are, respectively, proteins from control, Dex-induced and Zn^{2+} -induced cells in the absence of inhibitors. Lanes d, e and f are, respectively, proteins from control, Dex-induced and Zn^{2+} -induced cells in the presence of $1 \mu\text{g ml}^{-1}$ of actinomycin D during the 6-h induction period. Lanes g, h and i are, respectively, control Dex-induced and Zn^{2+} -induced cells in the presence of 10^{-4} M cycloheximide during the induction period. Once again, Dex and Zn^{2+} exposure leads to an elevation in translatable MT-mRNA (lanes a–c). Both the hormone- and metal-elicited elevations in MT-mRNA are inhibited by $1 \mu\text{g ml}^{-1}$ of actinomycin D (lanes d–f) but not by 10^{-4} M cycloheximide (lanes g, h, i). This concentration of cycloheximide blocks more than 90% of protein synthesis in HeLa cells (results not shown).

To quantitate the amounts of MT synthesized, a fluorogram prepared from the experiment shown in Fig. 2 was scanned with a densitometer. The optical density (a direct function of radioactivity, data not shown) in the MT band was summed and normalized to the amount of total radioactivity applied to the gel. Figure 3 shows the quantitative results from a densitometric analysis of a fluorogram from the same experiment shown in Fig. 2. In this particular experiment MT-mRNA was elevated about five-fold by 10^{-7} M Dex and six-fold by 3.7×10^{-5} M Zn^{2+} . Inclusion of $1 \mu\text{g ml}^{-1}$ actinomycin D in the culture medium during the induction period not only prevented the elevation of MT-mRNA, but also lowered its level relative to control cultures by 50–60%. In contrast, inclusion of 10^{-4} M cycloheximide did not inhibit the induction of MT-mRNA; in fact, cycloheximide caused an elevation in the level of MT-mRNA in both control and treated cultures.

We considered it essential to examine the role of RNA and protein synthesis in MT induction by Zn^{2+} and Dex at similar levels of induction. The Dex concentration used (10^{-7} M) causes maximal MT synthesis in response to this inducer. In contrast, we used a concentration of Zn^{2+} (3.7×10^{-5} M) which, although it causes approximately the same level of induction as maximal dexamethasone, results in only 20% of the maximal MT response to the optimal Zn^{2+} concentration (10^{-4} M). Variable cycloheximide enhancement of MT synthesis (40–100%) in Zn^{2+} -treated cultures and control cultures was observed in two *in vitro* translation experiments and four *in vivo* experiments of the type described in Fig. 1 legend. In contrast, we observed only 0–30% cycloheximide enhancement with Dex-treated cells. The stimulatory effect of cycloheximide observed in control- and Zn^{2+} -induced, but not Dex-induced expression of MT-mRNA may be due to the fact that in one condition (with Dex) the system is operating at maximal potential for that physiological condition, whereas in the other cases (control, Zn^{2+}) the system is operating in sub-optimal conditions of expression. Alternatively, the glucocorticoid receptor may have a short half life: its decay in the presence of cycloheximide may counterbalance a true stimulating effect of the drug. Recently, Enger *et al.*²² have also used *in vitro* translation of mRNA in a wheat-germ system to demonstrate that induction of MT in cultured CHO cells in response to cadmium occurs as a consequence of an elevation in translatable message.

Cox¹⁹ reported that exposure of HeLa cells to Dex leads to an increased uptake of Zn^{2+} . We find that Dex exposure of HeLa cells grown in serum-free medium also leads to a twofold elevation in the uptake of $^{65}\text{Zn}^{2+}$ after 24 h (Table 1, expt 1). Cox also reported the Dex-induced Zn^{2+} uptake was,

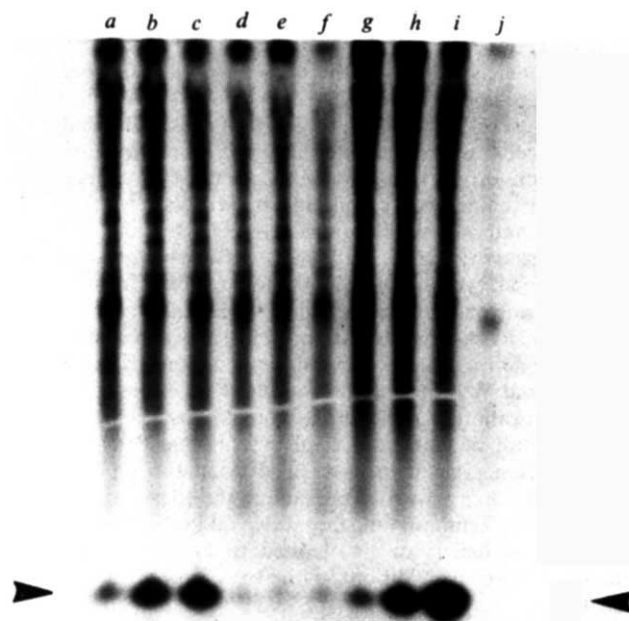


Fig. 2 *In vitro* translation of RNA preparations from control, Dex-treated and Zn^{2+} -treated HeLa cells and the effect of actinomycin D and cycloheximide on MT-mRNA accumulation. Confluent cultures of HeLa cells were incubated for 6 h in: a, control medium; b, medium containing 10^{-7} M Dex; c, medium containing 3.7×10^{-5} M Zn^{2+} ; d, control medium plus $1 \mu\text{g ml}^{-1}$ actinomycin D; e, 10^{-7} M Dex plus $1 \mu\text{g ml}^{-1}$ actinomycin D; f, 3.7×10^{-5} M Zn^{2+} plus $1 \mu\text{g ml}^{-1}$ actinomycin D; g, control medium plus 10^{-4} M cycloheximide; h, 10^{-7} M Dex plus 10^{-4} M cycloheximide; i, 3.7×10^{-5} M Zn^{2+} plus 10^{-4} M cycloheximide; j, no exogenous RNA added to the translation system. At the end of the 6-h incubation period, total cytoplasmic RNA was extracted and translated in the cell-free system. The translation products were reduced and alkylated, dried down under vacuum in a desiccator and resolubilized in 50 μl of electrophoresis sample buffer. A 10- μl aliquot containing 40,000–60,000 c.p.m. was subjected to electrophoresis on 20% polyacrylamide, 0.1% SDS gels. The position of the MT band on the resulting fluorogram is indicated by the arrows.

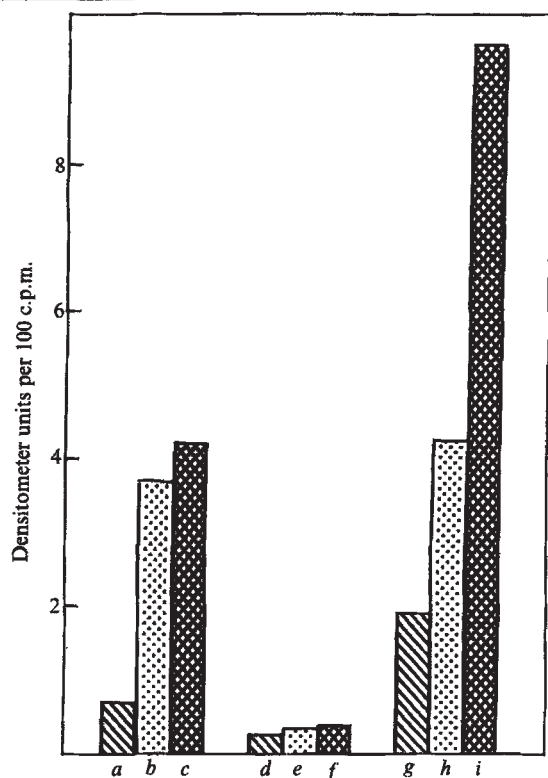


Fig. 3 Quantitation of the effects of RNA and protein synthesis inhibitors on elevation of MT-mRNA in response to Dex and Zn^{2+} . A fluorogram similar to that shown in Fig. 2, but in the linear response range, was scanned with an Optronics Photoscan model P-1000 digitalized densitometer, using a photometer window with a 200- μ m raster. RNA at 10 μ g per translation (a subsaturating concentration) was used. The density in the MT band was summed and normalized to the amount of radioactivity applied to each slot.

however, dependent on *de novo* RNA and protein synthesis¹⁹. We find that after 6 h of exposure to Dex there may already be a slight increase in $^{65}Zn^{2+}$ uptake. More importantly, in the presence of cycloheximide there is no Dex stimulation of $^{65}Zn^{2+}$ uptake over the 6-h induction period (Table 1, expts 2, 3). In contrast to Dex stimulation of Zn^{2+} transport, the induction of MT-mRNA is not blocked by cycloheximide (Figs 2, 3). In the HeLa system, glucocorticoid induction of MT is thus not a consequence of protein synthesis-dependent Zn^{2+} uptake into the cells, as suggested by Failla and Cousins¹⁷ for primary rat hepatocytes.

Several of the currently studied steroid hormone induction systems are not primary induction responses. Hepatic induction of $\alpha 2\mu$ -globulin mRNA by glucocorticoid hormones is inhibited by cycloheximide²³, in contrast to our results for glucocorticoid hormone induction of MT-mRNA. McKnight²⁴ has also reported that the induction in explants of chick oviduct of mRNA for both ovalbumin and conalbumin by oestrogen (or progesterone) is blocked by inhibition of protein synthesis. A primary induction system such as the Dex induction of MT synthesis in HeLa cells, in which no intervening protein synthesis need occur, should be much more amenable to cell-free analysis of transcription control by steroid hormones.

We conclude that (1) Dex and Zn^{2+} both lead to an elevation of the mRNA for MT in HeLa cells, (2) the elevation is dependent on *de novo* RNA synthesis (inhibition by actinomycin D), (3) the elevation is independent of *de novo* protein synthesis (insensitivity to cycloheximide), and (4) the elevation is not dependent on increased Zn^{2+} transport (Table 1). MT induction by both Dex and Zn^{2+} is a primary induction response¹⁸, that is, it is not dependent on the induced synthesis of any protein intermediates. The MT induction system should provide an excellent experimental

model both for the study of the physiological regulation by glucocorticoid hormones of Zn^{2+} metabolism and for the elucidation of the control mechanisms responsible for regulation of gene expression in eukaryotes by heavy metals and glucocorticoids.

This work was supported by DOE Contract DE AM03 76 SF00012 and EPA Contract IAG-D5-E-681-A0. Film scanning was performed on equipment purchased with NIH research funds (GM16925) and supported by the Parvin Cancer Research Laboratories core grant. Scanning and integration software was prepared by Richard G. Fisher.

Received 16 July 1979; accepted 16 April 1980.

- Margoshes, M. & Vallee, B. L. *J. Am. chem. Soc.* **79**, 4813-4814 (1957).
- Kagi, J. H. R. & Vallee, B. L. *J. biol. Chem.* **236**, 2435-2442 (1961).
- Sabbioni, F. & Marafante, E. *Environ. Physiol. Biochem.* **5**, 132-141 (1975).
- Wisniewska, J. M., Trojanowska, B., Piotrowski, J. K. & Jakubowski, M. *Toxicol. appl. Pharmac.* **16**, 754-763 (1970).
- Shaikh, Z. A. & Lucis, O. J. *Fedn Proc.* **29**, 298 (1970).
- Winge, D. R., Premakumar, R. & Rajagopalan, K. V. *Archs Biochem. Biophys.* **170**, 242-252 (1975).
- Squibb, K. S. & Cousins, R. J. *Environ. Physiol. Biochem.* **4**, 24-30 (1974).
- Squibb, K. S. & Cousins, R. J. *Biochem. biophys. Res. Commun.* **75**, 806-812 (1977).
- Webb, M. & Daniel, M. *Chem. biol. Interact.* **10**, 269-276 (1975).
- Failla, M. L. & Cousins, R. J. *Biochim. biophys. Acta* **543**, 293-304 (1978).
- Rudd, C. J. & Herschman, H. R. *Toxicol. appl. Pharmac.* **44**, 511-521 (1978).
- Rudd, C. J. & Herschman, H. R. *Toxicol. appl. Pharmac.* **47**, 271-276 (1979).
- Karin, M. & Herschman, H. R. *Science* **204**, 176-177 (1979).
- Eitzel, R. R., Shapiro, S. G. & Cousins, R. J. *Biochem. biophys. Res. Commun.* **89**, 1120-1126 (1979).
- Cox, R. P. *Molec. Pharmac.* **4**, 510-521 (1968).
- Cox, R. P. & Ruckenstein, A. J. *cell. Physiol.* **77**, 71-82 (1971).
- Failla, M. L. & Cousins, R. J. *Biochim. biophys. Acta* **538**, 435-444 (1978).
- Yamamoto, K. R. & Alberts, B. A. *Rev. Biochem.* **45**, 721-746 (1976).
- Cox, R. P. *Science* **165**, 196-199 (1969).
- Anderson, C. W., Lewis, J. B., Atkins, J. F. & Gesteland, R. F. *Proc. natn. Acad. Sci. U.S.A.* **71**, 2756-2760 (1974).
- Andersen, R. D. & Weser, U. *Biochem. J.* **175**, 841-852 (1978).
- Enger, M. D., Rall, L. B. & Hildebrand, C. E. *Nucleic Acids Res.* **7**, 271-288 (1979).
- Chen, C. C. & Feigelson, P. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2669-2673 (1979).
- McKnight, G. S. *Cell* **14**, 403-413 (1978).
- Laskey, R. A. & Mills, A. D. *Eur. J. Biochem.* **56**, 335-341 (1975).

Independent replication and expression of B-component RNA of cowpea mosaic virus

Rob Goldbach, Geertje Rezelman & Albert van Kammen

Department of Molecular Biology, Agricultural University, De Dreijen 11, 6703 BC Wageningen, The Netherlands

Cowpea mosaic virus (CPMV) is a plant virus with a split genome, composed of two single-stranded plus-type RNA molecules which are separately encapsidated in two (bottom and middle) nucleoprotein particles¹⁻³. The protein capsids of the bottom (B) and middle (M) component are similar and consist of two proteins in equimolar amounts^{4,5}. Both B and M components are necessary for virus multiplication, indicating that the genetic information is distributed between both (B and M) RNAs⁶. However, little is known about the location of genes on either RNA and the reason for a divided genome is unknown. Inoculation of cowpea protoplasts with complete virus (M+B components) results in the synthesis of at least seven virus-specific polypeptides, including the capsid proteins^{7,8}. We show here that on inoculation of protoplasts with the separated components, the B component RNA is replicated and expressed independently, whereas the M component RNA is not. The results presented suit a model in which B RNA codes for early functions and M RNA for late functions during viral infection, supplying a possible explanation for the divided genome of CPMV.

The molecular weights (MWs) of M and B RNAs are 1.37×10^6 and 2.02×10^6 , respectively⁹. Both RNAs possess a 3'-terminal poly(A) tail¹⁰ and a protein covalently linked to their

5' termini^{11,12}. Translation in a wheat-germ cell-free system and in extracts from rabbit reticulocytes results in the synthesis of large proteins which readily account for more than 80% of the coding capacity of both M and B RNA^{13,15}. Pelham¹⁵ has shown that in the reticulocyte system some processing of the large proteins occurs. However, no polypeptides corresponding to the viral coat proteins were observed. Mesophyll protoplasts can be isolated from the primary leaves of cowpea plants (*Vigna unguiculata* (L.) and infected with CPMV¹⁶. We have analysed the proteins synthesized in this *in vivo* system after inoculation with separated B and M components.

Figure 1 shows the electrophoretic patterns of the proteins from mock-infected protoplasts and protoplasts inoculated with M, B or the mixture of M+B components. In agreement with earlier results^{7,8}, protoplasts infected with M+B components produce seven virus-specific polypeptides of MWs 170,000, 110,000, 87,000, 84,000, 37,000, 30,000 and 23,000. The polypeptide pattern obtained from protoplasts infected with M component alone is similar to that of the uninfected protoplasts. No virus-specific proteins were detected. In contrast, protoplasts infected with B component alone produce all virus-specific polypeptides with the exception of the (37,000 and 23,000 MW) capsid proteins. No immunoprecipitate with antiserum against virions was obtained from protoplasts infected with either M or B components, confirming the absence of coat proteins. The total of the MWs of the five B RNA-coded polypeptides greatly exceeds the coding capacity of the RNA. Peptide fingerprint experiments, using *Staphylococcus aureus* V8 protease, have shown, however, that the polypeptides of MWs 110,000, 87,000 and 84,000 are derived from the 170,000 MW polypeptide by post-translational cleavage (data not shown). The results shown in Fig. 1 raise the question of why B RNA alone can be expressed whereas M RNA cannot. Two possible explanations can be given. First, M RNA can only be translated when B RNA or B RNA-coded proteins are present in the cell. This explanation seems improbable as M RNA is readily translated *in vitro*. Second, B RNA codes for the viral replicase^{17,18} and on infection of protoplasts with B component, B RNA is replicated to give a large quantity of RNA subsequently directing detectable translation. Translation of M RNA in protoplasts remains undetectable because multiplication of M RNA does not occur.

Table 1 Replicase activity in cowpea protoplasts inoculated with CPMV components

Protoplasts inoculated with	³ H-UTP incorporation per 13 × 10 ⁶ protoplasts (c.p.m.)
M	5,492
B	5,688
M+B	17,876
	17,332

Portions of 13 × 10⁶ protoplasts were inoculated with 3.5 µg M, 3.5 µg B or 7 µg (1:1 mixture) M+B components and incubated as described elsewhere⁷. The cells were collected 42 h after inoculation, resuspended in 1 ml of 50 mM Tris-HAc (pH 7.4), 10 mM KAc, 1 mM EDTA, 10 mM dithioerythritol (DTE) and 0.5 mM phenylmethylsulphonylfluoride (PMSF) and disrupted at 0°C in a Thomas homogenizer with a Teflon pestle. Cell homogenates were centrifuged at 1,000g for 15 min. The supernatants were adjusted to 20% (v/v) glycerol and centrifuged at 31,000g for 30 min. The 31,000g pellets were resuspended in 0.4 ml 50 mM Tris-HAc (pH 8.2), 25% (v/v) glycerol, 50 mM KAc, 1 mM EDTA, 10 mM DTE and 0.5 mM PMSF. ³H-UTP incorporation in the resuspended 31,000g pellets was measured in standard replicase assay conditions¹⁸. Although the ratio between the activities of uninfected and M+B-infected cells is only slightly more than 3, it was reproducible in other experiments. Moreover, in time course experiments (not shown) it was found that the extra activity in M+B-inoculated cells was induced at 15–30 h post-infection, that is, the period of maximal virus production¹⁶.

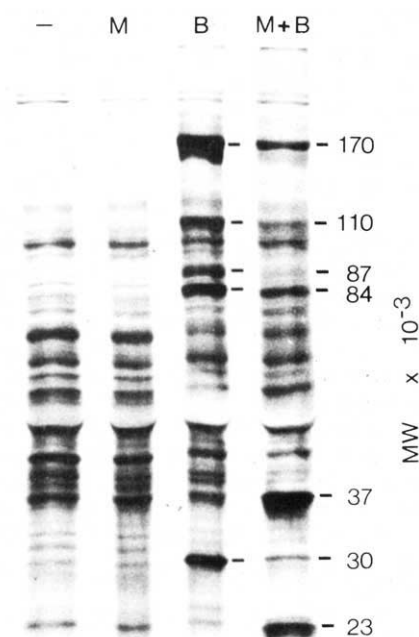


Fig. 1 Analysis on a 7–15% linear SDS-polyacrylamide gradient gel of ³⁵S-labelled proteins from uninfected protoplasts (–), protoplasts inoculated with purified M components (M), purified B component (B), and complete virus (M+B). CPMV was grown in *Vigna unguiculata* L. 'blackeye early ramshorn' and purified as previously described²⁷. M and B components were separated by three cycles of centrifugation in a linear 15–30% zonal sucrose gradient (Beckman Ti 15 rotor, 16 h, 23,000 r.p.m. at 10°C). By means of the local lesion infectivity assay¹⁶ it was determined that M components were free of B and that B components contained less than 0.2% M. Portions of protoplasts (5 ml, 5 × 10⁵ ml⁻¹) were mock-infected or inoculated with 7 µg M, 7 µg B or 14 µg (1:1 mixture) M+B virus components and labelled with 150 µCi ³⁵S-methionine 20–25 h after infection as described in detail elsewhere⁷. Neither actinomycin D nor any other inhibitor of host protein synthesis was added in this experiment. Soluble protein fractions (31,000g supernatants) were prepared and electrophoresed (using equal amounts of radioactivity) as previously described⁷. In the M+B slot the large (170,000–84,000 MW) virus-specific polypeptides are not as clearly visible as in the B slot, as most radioactivity is found in the capsid proteins (37,000 and 23,000 MW). MW markers used were: *Escherichia coli* RNA polymerase subunits (MWs 165,000 and 155,000), *E. coli* β-galactosidase (116,200), phosphorylase a (92,500), human transferrin (80,000), bovine serum albumin (68,000), catalase (57,500), γ-globulin (54,000 and 23,500), ovalbumin (46,000), lactate dehydrogenase (35,000) and tobacco mosaic virus coat protein (17,500).

To test the latter hypothesis, the RNA replicase activity was examined in infected and uninfected protoplasts using the assay conditions for CPMV replicase as described by Zabel *et al.*^{17,18}. The results presented in Table 1 show that in protoplasts infected with a mixture of M+B the ³H-UTP-incorporating activity increases three times over the activity measured in uninfected protoplasts. A similar increase in activity occurs in protoplasts infected with B component but not in those infected with M component. This finding strengthens the suggestion that B RNA contains information essential for viral RNA replication. This suggestion was substantiated by analysing the RNAs produced. Protoplasts inoculated with M, B or M+B components, or uninfected, were labelled by incubation with ³²PO₄ 20–25 h after inoculation and then analysed for RNA. As shown in Fig. 2, protoplasts infected with a mixture of M+B components

produce two RNAs which co-migrate with standard CPMV B RNA and M RNA. These RNAs are absent in uninfected protoplasts as well as in protoplasts infected with M component. However, protoplasts inoculated with B component efficiently produce ^{32}P -labelled B RNA. This finding verifies that B RNA replicates independently whereas M RNA does not.

The absence of M RNA (or fragments of M RNA) in protoplasts infected independently with M or B components was checked using cDNA made from B and M RNA. The results of the hybridization experiments presented in Table 2 show that M RNA is not synthesized in protoplasts inoculated with M component alone, whereas in protoplasts inoculated with the M+B mixture this RNA is readily detected. The results confirm that B RNA is made in protoplasts infected with B component alone. The amount of B RNA produced in these cells is comparable with the amount of this RNA in protoplasts infected with M+B, consistent with the independent expression of B as shown in Fig. 1. To a certain degree CPMV resembles another two-component virus, tobacco rattle virus (TRV). The large RNA of TRV is also able to replicate independently, whereas no coat protein is produced¹⁹. CPMV differs from TRV, however, in that inoculation of plants with B component does not result in infection detectable by local lesions.

The data obtained from the present experiments provide evidence that B RNA can be independently replicated, implying that this RNA codes for a protein involved in replication. Although in theory B RNA (but not M RNA) may code for a protein inducing the expression of a host gene, this possibility is very unlikely. A host gene must be proposed, which is 'sleeping' in normal (uninfected) conditions and acting harmfully during infection. It is plausible that B RNA codes for the viral replicase or a subunit of this enzyme. Zabel *et al.*^{17,18} have isolated CPMV RNA replicase from virus-infected cowpea leaves and have shown that such replicase preparations contain a protein which binds CPMV RNA specifically and is absent in control preparations from healthy leaves. Together, these findings favour the hypothesis that CPMV RNA carries information for a protein required for viral RNA replication. They do not support the idea that plant viral RNA is replicated by a host enzyme, as has been suggested²⁰⁻²².

The results shown here suggest that M RNA codes for both capsid proteins, these being the only viral proteins absent in protoplasts infected with B component. A similar conclusion is reached from complementation studies using particles from different CPMV isolates^{23,24}. The results are consistent with a model for the distribution of genes among the components of CPMV in which B RNA exclusively codes for early proteins (for example, the replicase or replicase subunit(s)) and M RNA for late proteins (for example, both capsid proteins). The usefulness of the split genome would therefore lie in its ability to regulate separately the expression of early and late genes.

Table 2 Hybridization of ^{32}P -labelled RNA from infected and uninfected protoplasts with cDNA made from B and M RNA

^{32}P -RNA from protoplasts inoculated with	Hybridization (c.p.m.) to		
	M cDNA	B cDNA	Calf thymus DNA
—	165	122	93
M	173	147	130
B	290	2,966	161
M+B	1,665	2,342	152

Protoplasts were incubated with ^{32}P -orthophosphate 20–25 h after inoculation. For details see Fig. 2 legend. Portions of 100,000 c.p.m. ^{32}P -RNA were hybridized to 1 μg of filter-bound DNA for 18 h in 0.45 M NaCl and 0.045 M Na-citrate (pH 7.0) at 65°C, essentially as described by R.G. *et al.*²⁵. Complementary DNA from purified M and B RNA (M cDNA and B cDNA, respectively) was made as previously described²⁰.

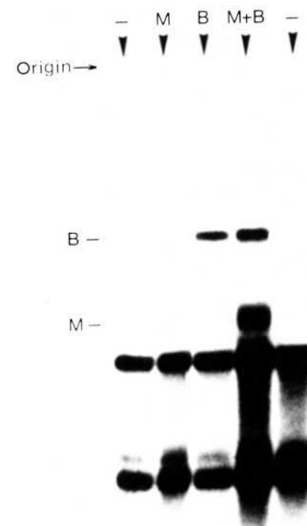


Fig. 2 Agarose gel electrophoresis of glyoxal-denatured RNAs from protoplasts infected with CPMV components. Portions of protoplasts (5 ml , $5 \times 10^5\text{ ml}^{-1}$) were inoculated with virus components as described in Fig. 1 legend. The cells were collected 20 h after inoculation, resuspended in phosphate-deficient medium⁷ ($10^6\text{ cells ml}^{-1}$) and labelled for 5 h with ^{32}P -orthophosphate (1 mCi ml^{-1}) in the presence of actinomycin D ($15\text{ }\mu\text{g ml}^{-1}$). Total cell RNA was isolated by heating the cells for 5 min at 60°C in a detergent mix (2% *p*-aminosalicylate, 1% Na-deoxycholate, 0.5% tri-isopropyl-naphthalene sulphate and 2% Sarkosyl in 50 mM Tris-HCl (pH 8.2) and 0.1 M NaCl) and centrifugation of the lysate on a 5.7 M CsCl cushion according to Glišin *et al.*²⁸. Yields were: uninfected cells, 840 μg (7,900 c.p.m. per μg); M-inoculated cells, 780 μg (8,200 c.p.m. per μg); B-inoculated cells, 860 μg (10,200 c.p.m. per μg); M+B inoculated cells, 790 μg (9,400 c.p.m. per μg); c.p.m. were measured by scintillation counting. RNA samples (comprising 20,000 c.p.m.) from uninfected (—) protoplasts and those inoculated with M, B and M+B components were denatured with glyoxal and electrophoresed in a 1.5% agarose gel according to McMaster and Carmichael²⁹. After the run the gel was dried and autoradiographed. B and M below the origin indicate the position of standard (unlabelled) virion B and M RNA in the gel. Recently, it has been reported³⁰ that the poly(A) tail of M RNA is heterogeneous in length (50–400 residues), whereas the length distribution of the poly(A) tail in B RNA shows a narrow distribution (50–150 residues). This heterogeneity is visible in the gel, where the band of B RNA is sharp but the band of M RNA rather diffuse.

We thank Drs J. Stanley and P. Zabel for helpful comments and Mr J. Verver for carrying out the RNA electrophoresis experiments. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

Received 15 February; accepted 29 April 1980.

1. Van Kammen, A. A. *Rev. Phytopath.* **10**, 125–150 (1972).
2. Jaspars, E. M. J. *Adv. Virus Res.* **19**, 37–149 (1974).
3. Bruening, G. in *Comprehensive Virology* Vol. 11 (eds Fraenkel-Conrat, H. & Wagner, R. R.) 55–141 (Plenum, New York, 1977).
4. Wu, G.-J. & Bruening, G. *Virology* **46**, 596–612 (1971).
5. Geelen, J. L. M. C., Van Kammen, A. & Verduin, B. J. M. *Virology* **49**, 205–213 (1972).
6. Van Kammen, A. *Virology* **34**, 312–318 (1968).
7. Rottier, P. J. M., Rezelman, G. & Van Kammen, A. *Virology* **92**, 299–309 (1979).
8. Rottier, P. J. M. thesis, Wageningen Agricultural Univ. (1980).
9. Reijnders, L., Aalbers, A. M. J., Van Kammen, A. & Thuring, R. W. J. *Virology* **60**, 515–521 (1974).
10. El Manna, M. M. & Bruening, G. *Virology* **56**, 198–206 (1973).
11. Stanley, J., Rottier, P., Davies, J. W., Zabel, P. & Van Kammen, A. *Nucleic Acids Res.* **5**, 4505–4522 (1978).
12. Daubert, S. D., Bruening, G. & Najarian, R. C. *Eur. J. Biochem.* **92**, 45–51 (1978).
13. Davies, J. W., Aalbers, A. M. J., Stuijk, E. J. & Van Kammen, A. *FEBS Lett.* **77**, 265–269 (1977).
14. Pelham, H. R. B. & Stuijk, E. J. *Proc. Coll. Nucleic Acids and Protein Synthesis in Plants*, CNRS, 691–695 (1977).
15. Pelham, H. R. B. *Virology* **96**, 463–477 (1979).
16. Hibi, T., Rezelman, G. & Van Kammen, A. *Virology* **64**, 308–318 (1975).
17. Zabel, P., Weenen-Swaans, H. & Van Kammen, A. *J. Virol.* **14**, 1049–1055 (1974).

18. Zabel, P., Jongen-Neven, I. & Van Kammen, A. *J. Virol.* **29**, 21–33 (1979).
19. Lister, R. M. *Proc. Fedn Am. Soc. exp. Biol.* **28**, 1875–1889 (1969).
20. Ikegami, M. & Fraenkel-Conrat, H. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2122–2124 (1978).
21. Ikegami, M. & Fraenkel-Conrat, H. *FEBS Lett.* **96**, 197–200 (1978).
22. Chiffot, S., Sommer, P., Hartmann, D., Stussi-Garand, C. & Hirth, L. *Virology* **100**, 91–100 (1980).
23. Gopo, J. M. & Frist, R. H. *Virology* **79**, 259–266 (1977).
24. Thongmookom, P. & Goodman, R. M. *Virology* **85**, 75–83 (1978).
25. Goldbach, R. W., Bollen-de Boer, J. E., Van Bruggen, E. F. J. & Borst, P. *Biochim. biophys. Acta* **521**, 187–197 (1978).
26. Davies, J. W., Verver, J. W. G., Goldbach, R. W. & Van Kammen, A. *Nucleic Acids Res.* **5**, 4643–4661 (1978).
27. Klootwijk, J., Klein, I., Zabel, P. & Van Kammen, A. *Cell* **11**, 73–82 (1977).
28. Gilišin, V., Crkvenjakov, R. & Byus, C. *Biochemistry* **13**, 2633–2637 (1974).
29. McMaster, G. K. & Carmichael, G. G. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4835–4838 (1977).
30. Ahlquist, P. & Kaesberg, P. *Nucleic Acids Res.* **7**, 1195–1203 (1979).

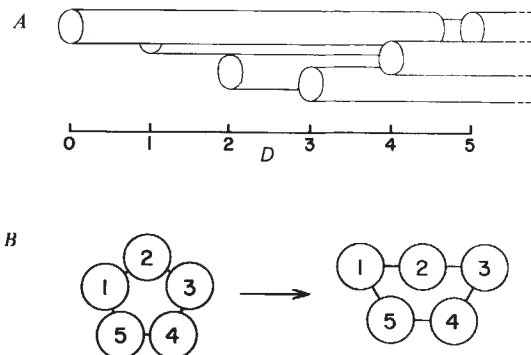


Fig. 1 Schematic representations of one end of a 5-fold microfibril of collagen molecules (A) and its conversion to a compressed microfibril where molecules in cross-section lie on a pseudo-hexagonal lattice (B). $D=67$ nm. The numbers designate the D segment cut in the cross-section. Molecules may be straight as shown or supercoiled.

Compressed microfibril models of the native collagen fibril

Benes L. Trus & Karl A. Piez

Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205

A three-dimensional crystal model for packing of collagen molecules (type I) in the native fibril has recently been proposed by Hulmes and Miller¹. It provides a straightforward explanation of the major features of the X-ray diffraction pattern, and is consistent with measurements of fibril density. However, there is independent evidence for a well defined microfibrillar substructure, which is absent from their model. This evidence, which is derived from electron microscopy^{2,5} and studies of *in vitro* assembly^{6,7}, the pattern of covalent cross-links⁸ and sequence analysis^{9,10}, is convincing. Therefore, we have searched for a means to reconcile this conflict. We now propose two models which contain five-stranded microfibrils compressed to place molecules (in cross-section) on a pseudo-hexagonal lattice. The unit cells are equivalent or related to the cell proposed by Hulmes and Miller¹. In the simplest case, molecules, and thus microfibrils, are straight-tilted. However, it is not ruled out that molecules are supercoiled and microfibrils are straight. Noncrystallographic considerations^{6,7,9,10} favour supercoiling.

The five-stranded microfibril in which D -staggered collagen molecules ($D=67$ nm) form a 5_1 or 5_4 helix (Fig. 1A), originally proposed by Smith¹¹, has been the most widely accepted model for packing of collagen molecules. In the original interpretation by Miller and colleagues^{12–14} of the X-ray diffraction pattern, the unit cell contained four 5-fold microfibrils in a tetragonal unit cell. A more recent study¹⁵ suggests a sheared tetragonal cell. In the present study we retain the microfibril but compress it to place molecules on a

pseudo-hexagonal lattice as illustrated in Fig. 1B. As proposed by Hulmes and Miller¹, we assume that the three strong X-ray diffraction reflections in the equatorial region with row-line spacings of $1/1.369$, $1/1.328$ and $1/1.264$ nm⁻¹ are the reciprocal distances between planes of molecules in the three sets that define the pseudo-hexagonal lattice. We suggest that a microfibril in isolation or in small bundles may have a symmetrical or loosely symmetrical 5-fold cross-section but that in large bundles (fibrils) the internal crystal energy is sufficient to distort the helix and force molecules onto a hexagonal lattice. Molecules may be straight or supercoiled, as discussed below. We envisage supercoiling as being accommodated by tilts of molecular segments, the angle varying about some average value. As a molecule coils about the microfibril axis it would appear at successive lattice points within the compressed microfibril cross-section. The trapezoidal cross-section, in general, would not rotate. The distortion required is consistent with NMR studies^{16–18} which have shown that since collagen molecules in the native fibril are in rapid segmental motion in directions perpendicular to the fibril axis, intermolecular interactions must be fluid. In addition, systematic as well as random deviations from the lattice are possible as long as a large portion of the protein density is near the lattice planes. That is, the actual structure of the microfibril may be between the two extremes illustrated in Fig. 1B.

In model 1 the compressed microfibrils are arranged in a hexagonal pattern as shown in Fig. 2. Every point on the lattice of molecules is occupied and the unit cell contains a D segment of one microfibril. The unit cell is equivalent to that proposed by Hulmes and Miller¹, but the arrangement of molecules within the cell is different. Table 1 shows the agreement between observed and calculated row-line spacings. As can be seen in Fig. 2, the unit cell can be drawn in two equivalent ways, one of which was selected by Hulmes and Miller¹. We have used the other because it is directly related to the unit cell of model 2.

The simplest way to explain the equatorial position of the $1/1.264$ -nm⁻¹ reflection and the off-equatorial positions of the $1/1.328$ - and $1/1.369$ -nm⁻¹ reflections is to straight-tilt molecules in the direction of the 1.264 -nm plane so the tilting vector is 0° normal to that plane but about 4° normal to the other two planes. Here also, the unit cell of our model 1 is equivalent to the Hulmes and Miller¹ cell and thus provides as satisfactory an explanation of the X-ray diffraction pattern insofar as the analysis has been developed. However, we do not rule out the possibility that molecules are supercoiled about the microfibril axis rather than straight-tilted. The strong reflections near $1/1.3$ nm⁻¹ would then be explained by sampling of the transform of the supercoiled molecules. Because distorted helices are involved, this more complex

Table 1 Observed and calculated equatorial row-line spacings

Observed	Model 1	Model 2	
		Smaller cell	Larger cell
3.800	3.777 (1, 0)	3.777 (1, 0)	3.777 (1, 1)
		3.837 (1, -1)	3.837 (2, 0)
2.651	2.582 (1, -1)	2.582 (1, -2)	2.621 (1, -2)
2.461	2.436 (0, 1)	2.436 (0, 2)	2.436 (2, -2)
1.890	1.889 (2, 0)	1.889 (2, 0)	1.889 (2, 2)
1.748	1.748 (1, 1)	1.748 (1, 2)	1.748 (1, -3)
1.57	—	1.560 (2, 1)	1.560 (1, 3)
1.503	—	—	1.505 (5, -2)
1.369	1.369 (3, -1)	1.369 (3, -2)	1.369 (5, 1)
1.328	1.328 (1, -2)	1.328 (1, -4)	1.328 (6, -1)
1.264	1.264 (2, 1)	1.264 (2, 2)	1.264 (0, 4)

For convenience real spacings are given. Units are nanometres. Indices are shown in parentheses. Observed row-line spacings are from rat tail tendon^{12–14}. For descriptions of models 1 and 2, see Figs 2 and 3, respectively.

situation requires a more complex analysis, which we have not attempted.

The advantage of supercoiled molecules is that the microfibrils are better defined and thus provide a better explanation of evidence for a microfibrillar substructure, already quoted. For example, *in vitro* studies^{6,7} have shown that assembly of native fibrils from collagen monomer is a stepwise process involving thin filaments as intermediates. Lateral association of supercoiled microfibrils is consistent with these data whereas assembly of straight-tilted molecules implies a three-dimensional crystallization. Also, regularities in the amino acid sequence can be explained by supercoiling of molecules in a microfibril^{9,10}.

In model 2 we assume that the asymmetric unit contains *D* segments of two compressed microfibrils related by a noncrystallographic 2-fold axis. We have examined all possible arrangements and find one (Fig. 3A) in which the unit cell (Fig. 3B, smaller cell) accurately predicts the observed reflections in the near-equatorial region of the X-ray diffraction pattern (Table 1). The unit cell contains one asymmetric unit and its dimensions, not surprisingly in retrospect, are the same as those of the unit cell in model 1 except that one dimension is doubled. The advantage of model 2 is that it predicts one of two observed weak reflections not predicted by model 1, and it predicts a second reflection near the critical reflection at $1/3.8\text{ nm}^{-1}$ (Table 1). Both predicted reflections near $1/3.8\text{ nm}^{-1}$ arise from important structural features of the model (Fig. 3B) and thus both could be present but unresolved.

If the unit cell in model 2 is doubled (Fig. 3B), the second of the two reflections not predicted by model 1 is present and the fit to the observed reflection at $1/2.651\text{ nm}^{-1}$ is markedly improved (Table 1, larger cell). That is, the true unit cell may contain four compressed microfibrils. If so, they must in some sense be non-equivalent. The situation may be analogous to the earlier interpretation¹²⁻¹⁴ of the X-ray diffraction data in which the unit cell contained four 5-fold microfibrils related by a 4-fold screw axis. Therefore, some of the arguments based on that model may still be valid. For example, the larger unit cell is consistent with recent electron microscopic evidence⁵ for an 8-nm unit in collagen fibrils. Regularities in the amino acid sequence have also been interpreted in terms of a 4-fold screw axis^{9,10}, results which are not inconsistent with the distortion required for hexagonal packing of supercoiled molecules.

As in model 1, molecules in model 2 may be straight-tilted or supercoiled. The same arguments hold. A somewhat stronger case may be made for supercoiling in model 2 as the tilting vectors arising from the supercoil will be largely parallel to the long axes of the compressed microfibril cross-sections, which lie in the 1.264-nm planes. As already noted, this spacing gives rise to an equatorial reflection whereas the 1.369-

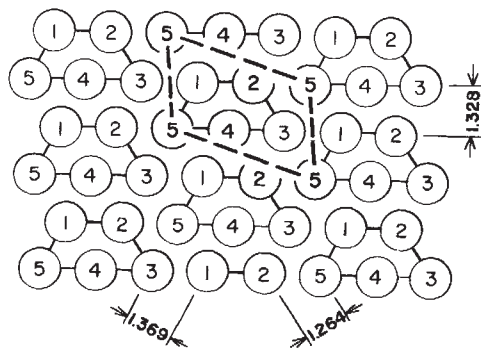


Fig. 2 Model 1. One possible arrangement of molecules in a native collagen fibril. The lines join sets of five molecules constituting a microfibril (Fig. 1). The lattice spacings are given in nanometres. The unit cell (dashed lines) has dimensions $a = 2.668\text{ nm}$, $b = 4.136\text{ nm}$ and $\gamma = 114.04^\circ$ and contains one microfibril.

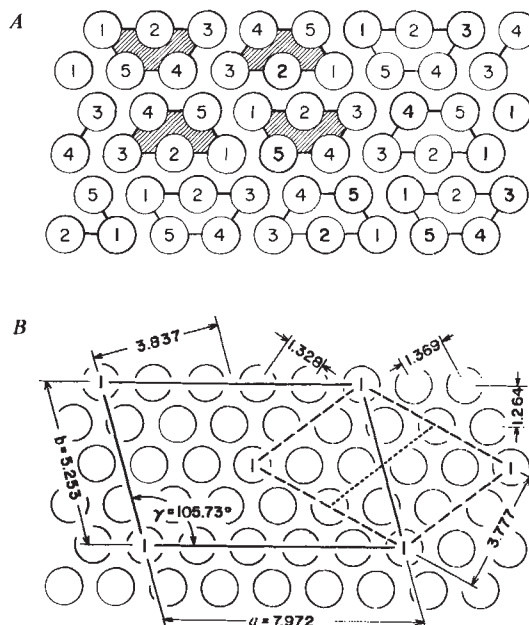


Fig. 3 Model 2. A, A second possible arrangement of molecules in a native collagen fibril. The lines join sets of five molecules constituting a microfibril (Fig. 1). The shading designates a group of four microfibrils which in the larger unit cell constitute the true repeating unit. B, The same arrangement showing the larger (solid lines) and smaller (dashed lines) unit cells, some of the major spacings and the dimensions of the larger unit cell. Distances are given in nanometres. The dimensions of the smaller unit cell are $a = 5.335\text{ nm}$, $b = 4.136\text{ nm}$ and $\gamma = 114.04^\circ$. The dotted line halves the smaller cell and defines a pseudo-cell identical to the unit cell of model 1.

and 1.328-nm planes give rise to reflections that are off-equatorial. However, a detailed analysis has not been done.

Of course, the unit cell in model 1 can be doubled or quadrupled and microfibrils made non-equivalent; the unit cells of models 1 and 2 become identical. Therefore, evidence for a large unit cell does not favour one model over the other. It is also possible that different arrangements of microfibrils with varying degrees of order exist within a group of fibrils.

Further analysis and perhaps independent evidence will be needed to provide a definitive answer. What we have shown is that hexagonal packing of molecules is not inconsistent with some type of ordered microfibrillar substructure.

We thank Drs Hulmes and Miller for a copy of their manuscript¹ before its publication.

Note added in proof: We have recently learned that Katz and Li¹⁹ in 1973 proposed the general idea that microfibrillar structures (5- and 7-stranded) could be contained within a hexagonal lattice of collagen molecules.

Received 24 January; accepted 2 May 1980.

- Hulmes, D. J. & Miller, A. *Nature* **282**, 878-880 (1979).
- Piez, K. A. & Miller, A. *J. supramolec. Struct.* **2**, 121-137 (1974).
- Doyle, B. B. *et al. Proc. R. Soc. B* **186**, 67-74 (1974).
- Ruggeri, A., Benazzo, F. & Reale, E. *J. ultrastruct. Res.* **68**, 101-108 (1979).
- Parry, D. A. D. & Craig, A. A. *Nature* **282**, 213-215 (1979).
- Gelman, R. A., Williams, B. R. & Piez, K. A. *J. biol. Chem.* **254**, 180-186 (1979).
- Gelman, R. A. & Piez, K. A. *J. biol. Chem.* (in the press).
- Light, N. D. & Bailey, A. J. *Biochem. J.* **185**, 373-381 (1980).
- Piez, K. A. & Trus, B. L. *J. molec. Biol.* **122**, 419-432 (1978).
- Traub, W. *FEBS Lett.* **92**, 114-120 (1978).
- Smith, J. W. *Nature*, **219**, 157-158 (1968).
- Miller, A. & Wray, J. S. *Nature* **230**, 437-439 (1979).
- Miller, A. & Parry, D. A. D. *J. molec. Biol.* **75**, 441-447 (1973).
- Fraser, R. D. B., Miller, A. & Parry, D. A. D. *J. molec. Biol.* **83**, 281-283 (1974).
- Fraser, R. D. B. & MacRae, T. P. *J. molec. Biol.* **127**, 127-133 (1979).
- Torchia, D. A. & Vanderhart, D. L. *J. molec. Biol.* **104**, 315-321 (1976).
- Jelinski, L. W. & Torchia, D. A. *J. molec. Biol.* **133**, 45-65 (1979); *J. molec. Biol.* **138**, 255-272 (1980).
- Jelinski, L. W., Sullivan, C. E. & Torchia, D. A. *Nature* **284**, 531-534 (1980).
- Katz, F. P. & Li, S. H. *J. molec. Biol.* **73**, 351-369 (1973).

Structure of cytochrome *c'*: a dimeric, high-spin haem protein

Patricia C. Weber*§, R. G. Bartsch†, M. A. Cusanovich*, R. C. Hamlin‡, A. Howard‡, S. R. Jordan*, M. D. Kamen†, T. E. Meyer†, D. W. Weatherford*, Nguyen huu Xuong† & F. R. Salemme*

*Departments of Biochemistry and Chemistry, New Chemistry Building, University of Arizona, Tucson, Arizona 85721

†Department of Chemistry A-002, University of California at San Diego, La Jolla, California 92093

‡Departments of Physics, Chemistry and Biology, University of California at San Diego, La Jolla, California 92093

§To whom correspondence should be addressed

The bacterial cytochromes *c'* (ref. 1) occur in various photosynthetic and denitrifying bacteria, where they are presumed to function in electron transport². Most cytochromes *c'* are isolated as dimeric molecules composed of two identical subunits of ~14,000 molecular weight (MW), with each polypeptide chain incorporating a protohaem IX prosthetic group covalently bound through thioether linkages formed by condensation of two cysteine side chains with the haem vinyl groups³. Although a similar mode of covalent haem attachment is found in members of the well studied mitochondrial cytochrome *c* family⁴, the cytochromes *c'* are, in contrast, high-spin haem proteins (hence the prime designation) which bind neutral ligands⁵, characteristic properties generally associated with members of the oxygen-binding globin family⁶. Here we present a preliminary structural description of the cytochrome *c'* derived from the photosynthetic, purple non-sulphur bacterium *Rhodospirillum molischianum*, and show that it bears little structural resemblance to members of either the cytochrome *c* or globin structural families. The cytochrome *c'* monomer structure is, instead, principally organized as a left-twisted, 4- α -helical bundle, a structural motif previously observed in several other sequentially and functionally unrelated proteins^{7,13}.

R. molischianum cytochrome *c'* crystallizes in the orthorhombic space group $P2_12_12_1$ ($a=56.5$ Å, $b=71.8$ Å, $c=75.6$ Å) with a dimeric molecule (MW ~29,000) in the crystallographic asymmetric unit¹⁴. Native and heavy atom (K_2PtCl_6 and K_2HgI_4) intensity data were collected from three crystals on the multiwire area detector developed by Xuong and co-workers at the University of California at San Diego^{15,16}. Heavy atom sites for the singly substituted platinum and doubly substituted mercury derivatives were

Table 1 Data collection and phase refinement for *R. molischianum* cytochrome *c'*

Crystal	Native	K_2PtCl_6 *	K_2HgI_4 †
No. of intensities measured (observations/unique reflections)	81,380/18,513	71,072/10,881	67,994/14,082
Scaling <i>R</i>	0.062	0.074	0.068
No. of reflections used in phase refinement	10,667	10,372	10,540
<i>R_C</i>	—	0.55	0.54
<i>R_K</i>	—	0.084	0.095
<i>E</i> / <i>F_H</i>	—	0.37	0.48
<i>E''</i> / <i>E</i>	—	0.23	0.25

The overall figure of merit, *m*, to 2.5 Å resolution is 0.72. *E*, *E''* = r.m.s. differences between observed and calculated heavy atom and anomalous dispersion contributions, respectively. *F_H* = mean heavy atom contribution.

*Platinum site at $x/a=0.402$, $y/b=0.236$, $z/c=0.144$, $B=16.6$ Å².

†Mercury site 1 at $x/a=0.082$, $y/b=0.073$, $z/c=0.061$, $B=32.0$ Å², and mercury site 2 at $x/a=0.253$, $y/b=0.321$, $z/c=0.386$, $B=40.1$ Å².

$$R_C = \frac{\sum |F_{PH} \pm F_P| - F_H(\text{calc})}{\sum |F_{PH} \pm F_P|} \text{ for centric reflections.}$$

$$R_K = \frac{\sum |F_{PH}(\text{obs}) - F_{PH}(\text{calc})|}{F_{PH}(\text{obs})}$$

determined by deconvolution of the respective heavy atom difference Patterson maps and referred to a common origin by calculating a difference Fourier map for each derivative based on phases derived from the other. Anomalous dispersion effects arising from the platinum and mercury derivatives were incorporated into the final phase refinement cycles (Table 1) as described by Matthews¹⁷, giving an overall figure of merit of 0.72 for the 10,667 independent reflections phased to 2.5 Å resolution. The resulting map was contoured to allow construction of a 2 cm Å⁻¹ Kendrew model of the dimer, which was built according to the established amino acid sequence of the molecule¹⁸. Preliminary atomic coordinates were measured from the model using an electro-mechanical optical superpositioning device¹⁹. Figure 1 shows a representative view of the electron density of the 2.5 Å resolution map, whose overall quality was sufficient to trace unambiguously the backbone chain and establish the majority of the side-chain conformations.

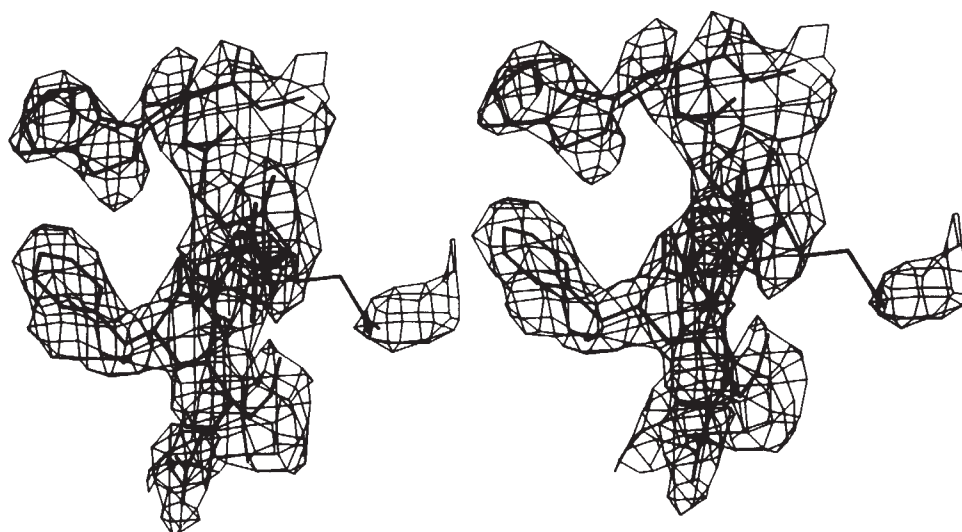


Fig. 1 Stereoscopic view of a segment of the C helix (residues Glu 81, Phe, Leu, Glu, Gly, Trp 86) superimposed on the 2.5 Å resolution MIR phased electron density map, showing a representative fit of the measured model coordinates. Prominent in the density are two aromatic residues situated in the protein interior and an external, solvent-exposed glutamate whose side chain is only partially defined at the present contour level which includes 15% of the unit cell volume.

Fig. 2 Schematic representation of the *R. molischianum* cytochrome *c'* dimer viewed down the molecular diad axis. The cytochrome *c'* monomers are principally composed of four, roughly parallel α -helices shown here as sequentially labelled cylinders. The left-twisted 4- α -helical arrangement of the monomers is qualitatively repeated at the subunit interface, due to the pairwise interaction of two helices from each subunit. The haem groups, situated at one end of the helical bundle, are orientated roughly parallel to each other and to the subunit interface, with their propionate side chains pointed towards the molecular surface. Regions of essentially extended polypeptide chain (shaded) connect and terminate the helices in each monomer. Approximately one-half of the residues in the molecule exist in an α -helical conformation.

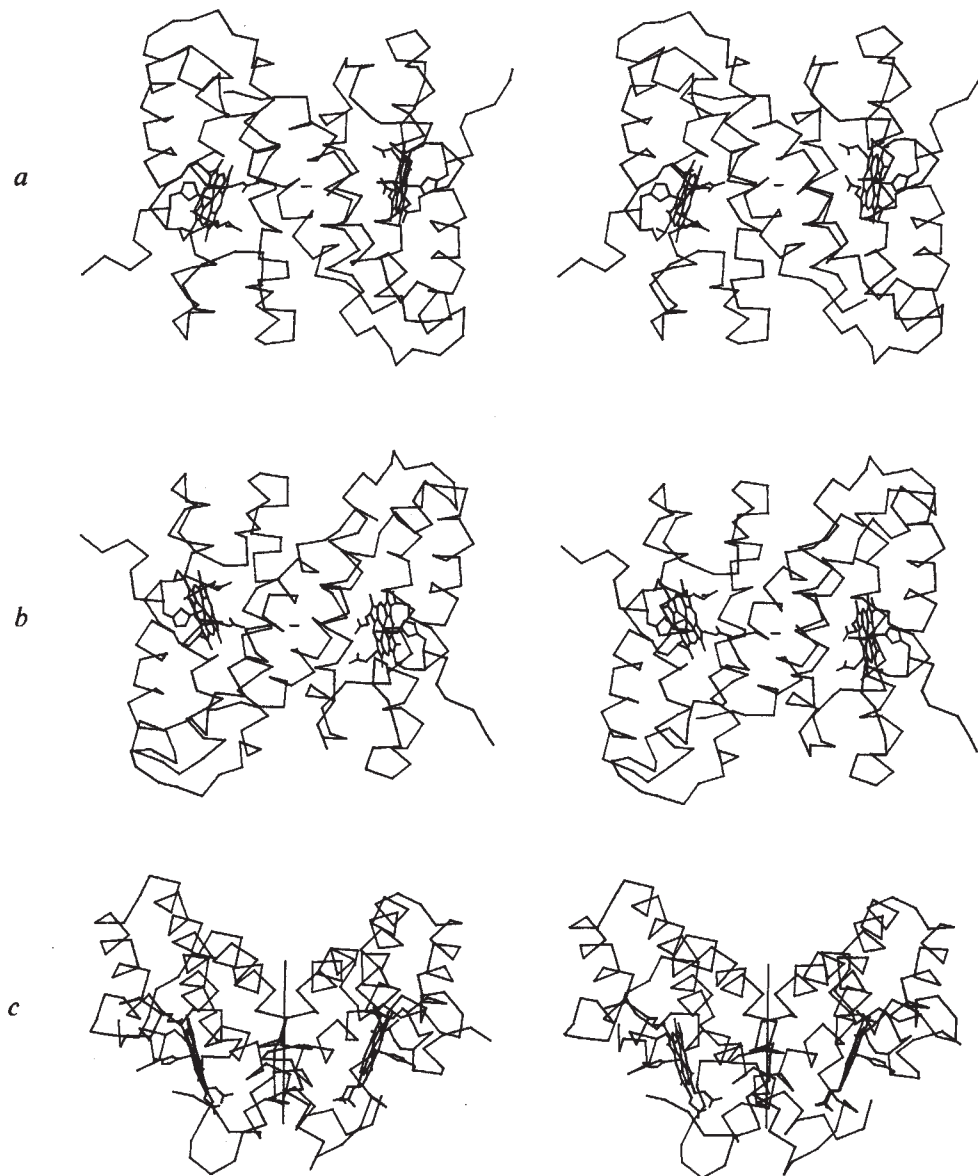
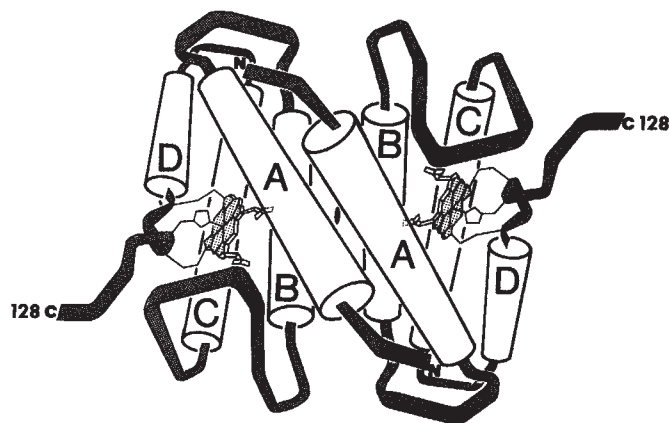


Fig. 3 Stereoscopic α -carbon drawings of *R. molischianum* cytochrome *c'*. *a* Shows the structure from the same aspect as Fig. 2, looking down the molecular diad axis at the molecular surface which has greatest haem exposure. The haem prosthetic groups, covalently bound near the carboxy termini, are separated in space by the two A helices, one from each subunit, which form the closest inter-helical interaction in the entire structure. The regions of extended polypeptide between helices are orientated towards this 'haem side' of the dimer. In contrast, the opposite side shown in *b* is primarily composed of α -helices. The $\sim 110^\circ$ crossing angle between subunits gives the dimer a V-shaped appearance when viewed normal to the diad axis as in *c*.

Figures 2 and 3 show schematic and α -carbon stereo representations of the cytochrome *c'* dimer. Each subunit is composed of a 128-amino acid polypeptide chain incorporating a covalently bound protohaem IX prosthetic group. Structurally, each monomer is composed of four, roughly parallel α -helices (sequentially labelled A-D) connected by more or less extended loops which join adjacent helix ends. Additional regions of essentially extended polypeptide chain occur at both the amino and carboxy termini of the molecule, the latter providing the covalent attachment points for the haem prosthetic group. The α -helices

of the monomer pack with an overall left-handed twist along the direction of the helix axes. However, the helices of the bundle distinctly diverge at one end to accommodate the haem prosthetic group.

The cytochrome *c'* monomers pack about a non-crystallographic diad axis at an angle between bundle axes of approximately 110° , giving the dimer a V-shaped appearance when viewed normal to the diad symmetry axis (Fig. 3c). The subunit interaction is mediated by the pairwise interaction of the A and B helices of each monomer, to give a 4- α -helical, left-twisted AA'-BB' bundle at the dimer interface whose

geometry is qualitatively similar to the arrangement of the helices within each monomer. The fact that the subunit interface interactions are similar to those within each monomer, both with respect to the hydrophobic nature of the amino acid residues involved and the overall similarity in extent and geometry of the α -helical interactions, provides an explanation for the observation that some homologous species of cytochrome *c*' can only be dissociated in conditions which lead to protein denaturation²⁰.

The protohaem IX prosthetic group in cytochrome *c*' forms three covalent attachments with the polypeptide chain, all of which are derived from the pentapeptide sequence Cys-X-X-Cys-His at residues 118–122. Two of these are the haem thioether linkages which serve to orientate the haem so that its propionate-substituted edge lies roughly parallel to each monomer bundle axis, and roughly perpendicular to the dimer diad axis. The third haem–polypeptide interaction is formed by the fifth position axial ligation of the ferric haem iron with N^ε2 of His 122. The sixth ligand ferric iron coordination site is either unoccupied or ligated by a solvent molecule, consistent with the high-spin character of the haem iron^{21,22}. Space-filling representations of the molecular structure show the haem environment to be highly asymmetric, with the imidazole side chain of the fifth haem iron ligand and two-thirds of the corresponding haem surface being exposed to the solvent. In contrast, the distal haem surface, which faces towards the dimer interface, is surrounded by aromatic or otherwise hydrophobic amino acid residues. Many of these packing interactions are provided by residues of the A helix, which are orientated so as to restrict access of exogenous ligands to the sixth haem iron coordination site through the most direct route to the molecular surface.

Because of the geometry of the monomer–monomer packing interaction, both haem groups of the molecule are situated on the same face of the dimer in nearly parallel orientation. Although this structural arrangement suggests that the haems of cytochrome *c*' may be situated so as to allow their simultaneous interaction with some physiological oxidoreductase, it is equally possible that this arrangement provides the basis for inter-haem communication in the molecule. Of relevance to the latter possibility is the fact that the 24 Å iron–iron vector is nearly coincident with the A–A' inter-helix contact normal vector, which at 6.0 Å is the shortest inter-helical interaction in the entire molecule. As described above, it is residues of the A helix which principally provide the packing interactions about the unliganded, distal haem surface. Thus, it seems possible that any local structural alterations accompanying haem ligation or oxidation could be communicated between the haems due to the intimacy of the A–A' helix packing interaction.

The principal similarity between the cytochromes *c*' and the mitochondrial cytochromes *c* is the common presence of a Cys-X-X-Cys-His sequence which provides the covalent attachment points and fifth axial ligand for the haem prosthetic group. Otherwise, the molecules are distinctly different with respect to overall chain folding, sequential location of the haem-binding peptide, spin state and extent of haem exposure to the solvent. Similarly, almost the only major feature shared by the cytochromes *c*' and the globins is their high-spin haem character, the general nature of the haem environments being otherwise quite different in the two molecules. Nevertheless, the fact that the cytochromes *c*' and globins are both high-spin haem proteins which can bind exogenous ligands and occur in both monomeric and higher aggregate forms^{2,6}, suggests interesting parallels between these molecules which require further investigation.

This work and facilities were supported by NIH grants GM18528-11 to M.D.K., RCD404EY0013 to M.A.C., GM20102, RR00757, to N.H.X., and GM21534, GM25664 to F.R.S. and NSF grants GB36019X to M.D.K., PCM7804349 to M.A.C. and PCM7722495 to N.H.X. Structure determination was done in partial fulfilment of the

requirements for a PhD at the University of Arizona by P.C.W. who was a Carl S. Marvel Fellow. F.R.S. received a Dreyfus Teacher-Scholar Award and the University of Arizona Computer Center also provided support.

Received 6 February; accepted 25 April 1980.

- Vernon, L. P. & Kamen, M. D. *J. biol. Chem.* **211**, 643–675 (1954).
- Bartsch, R. G. in *The Photosynthetic Bacteria* (eds Clayton, R. K. & Sistrom, W. R.) 249–279 (Plenum, New York, 1978).
- Barrett, J. & Kamen, M. D. *Biochim. biophys. Acta* **50**, 573–575 (1961).
- Salemme, F. R. *A. Rev. Biochem.* **46**, 299–329 (1977).
- Taniguchi, S. & Kamen, M. D. *Biochim. biophys. Acta* **74**, 438–455 (1963).
- Perutz, M. F. *A. Rev. Biochem.* **48**, 327–386 (1979).
- Bloomer, A. C., Champness, J. N., Bricogne, G., Staden, R. & Klug, A. *Nature* **276**, 362–368 (1978).
- Stubbs, G., Warren, S. & Holmes, K. *Nature* **267**, 216–221 (1977).
- Stenkamp, R. E., Sieker, L. C., Jensen, L. H. & McQueen, J. F. *Jr Biochemistry* **17**, 2499–2504 (1978).
- Ward, K. B., Hendrickson, W. A. & Klippenstein, G. L. *Nature* **257**, 818–821 (1975).
- Hendrickson, W. A., Klippenstein, G. L. & Ward, K. B. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2160–2164 (1975).
- Banyard, S. H., Stammers, D. K. & Harrison, P. M. *Nature* **271**, 282–284 (1978).
- Mathews, F. S., Bethge, P. H. & Czerwinski, E. W. *J. biol. Chem.* **254**, 1699–1706 (1979).
- Weber, P. & Salemme, F. R. *J. molec. Biol.* **117**, 815–820 (1977).
- Cork, C., Hamlin, R. C., Vernon, W., Xuong, Ng. H. & Perez-Mendez, V. *Acta crystallogr.* **A31**, 702–703 (1975).
- Xuong, Ng. H., Freer, S. T., Hamlin, R., Neilson, C. & Vernon, W. *Acta crystallogr.* **A34**, 289–296 (1978).
- Mathews, B. W. *Acta crystallogr.* **20**, 82–86 (1966).
- Amber, R. P. in *Abstr. 3rd int. Symp. Photosynthetic Prokaryotes* (ed. J. M. Nichols) F17 (Oxford, 1979).
- Salemme, F. R. & Fehr, D. G. *J. molec. Biol.* **70**, 697–700 (1972).
- Cusanovich, M. A. *Biochim. biophys. Acta* **236**, 238–241 (1971).
- Ehrenberg, A. & Kamen, M. D. *Biochim. biophys. Acta* **102**, 333–340 (1965).
- Maltempo, M. M., Moss, T. H. & Cusanovich, M. A. *Biochim. biophys. Acta* **342**, 290–305 (1974).

Molecular dynamics of ferrocycytochrome *c*

Scott H. Northrup, Michael R. Pear & J. Andrew McCammon*

Department of Chemistry, University of Houston, Houston, Texas 77004

Martin Karplus

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

The cytochromes *c* function as single-electron carriers in the mitochondrial electron transport chain^{1,2}. The native structures of the proteins from different species are quite similar^{1,2}, as are the structures of the reduced and oxidized forms^{3,4}. The 103-residue polypeptide chain of tuna cytochrome *c* contains 5 α -helical segments (residues 2–13, 50–54, 61–69, 71–74, 89–100); the haem group is almost completely buried in a hydrophobic pocket and is covalently bonded to the polypeptide chain by thioether linkages involving Cys 14 and Cys 17 and by linkages to the iron involving His 18 and Met 80 (refs 1–3). NMR^{5,6} and hydrogen exchange^{7,8} studies indicate significant internal mobility in cytochrome *c*. In spite of the apparent similarity of the time-average structures of the reduced and oxidized proteins, the structural fluctuations are significantly larger in the latter form^{2,7,8}. It has been suggested that the internal motions have a role in the electron transfer mechanism^{2,9}. A 16-ps computer simulation^{10–14} of the atomic motions in reduced tuna cytochrome *c* has now been completed; this reveals various correlations between the magnitudes of the atomic position fluctuations and the structural features of the protein.

The dynamical simulation method used here is similar to that used in earlier studies of the pancreatic trypsin inhibitor¹⁰. The protein potential energy function consists of a sum of terms associated with bonds, bond angles, dihedral angles, hydrogen bonds and nonbonded (van der Waals and electrostatic) interactions. The partial charges of the atoms have been reduced according to the distances of the atoms from the protein centre to approximate dielectric screening effects due to surrounding water¹⁵. To begin the simulation, the protein was dynamically equilibrated as follows. The X-ray structure^{16,17} was first subjected to 100 cycles of steepest descent energy minimization¹⁸ to relax any sizeable local stresses. The atoms were then assigned random maxwellian

*To whom reprint requests should be addressed.

velocities corresponding to a temperature of 50 K and the classical equations of motion were solved for a 2-ps period using the fifth order Gear algorithm¹⁰. Repeated velocity assignments corresponding to increasing temperatures followed by 2-ps periods of dynamical relaxation were used to bring the protein to a final average temperature of 297 K; the total period of temperature adjustment was 12 ps. To equilibrate the protein further, the dynamics was continued for 20 ps; the average temperature remained at 297 K. The actual simulation was obtained by continuing the run for an additional 16 ps.

The time-average structure from the simulation is quite similar to the 2.0-Å resolution X-ray structure¹⁶, except that several exposed, flexible sidechains have folded down on to the protein surface. The average r.m.s. deviation of C α positions is 1.58 Å, while that for all heavy atoms is 1.90 Å. There is some correlation between the residues with the largest deviations and those which have somewhat different positions in different crystal structures³. The largest deviations occur at the protein surface, particularly in the exposed sidechains of Lys 25, 27, 39, 72, 73 and 87 and Arg 38, where some atoms moved nearly 9 Å. Concomitant with these sidechain shifts, the accessible contact surface area of the protein as determined by Richards' method¹⁹ with a probe of 1.4-Å radius decreased from 1,751 Å² for the X-ray structure to 1,440 Å² for the dynamical average structure.

The fluctuations of the atoms about their average positions are sizeable and show interesting correlations with the time-average structure. The average r.m.s. fluctuation of the C α positions is 0.70 Å and that of all heavy atoms is 0.85 Å. To compare interior and exterior fluctuations, averages were

calculated for atoms in spherical shells of 3-Å thickness centred at the protein centre of mass. Shells with outer radii greater than 12 Å contain exterior atoms, because the average atomic density decreases rapidly with increasing radius for such shells. In the interior, the fluctuations are relatively small and increase slowly with shell size; the average r.m.s. fluctuations are 0.66, 0.70 and 0.73 Å for shells with outer radii of 6, 9 and 12 Å, respectively. In the exterior, the corresponding results are 0.98, 1.05 and 1.64 Å for shells with outer radii of 15, 18 and 21 Å, respectively. The average r.m.s. fluctuations for individual residues are correlated with their relative surface exposures in the time-average structure, with more exposed residues exhibiting larger fluctuations (Fig. 1a,c).

The haem group is expected to be relatively rigid due to the extensive pi electron delocalization in its ring system. The average r.m.s. fluctuation of the haem atom positions is 0.51 Å, which is significantly smaller than that of the other atoms in the protein interior. To assess the effect of the haem group on the mobility of nearby polypeptide atoms, a haem neighbourhood was defined by two cylindrical disks of 6-Å radius and 6-Å thickness lying on either side of the haem and coaxial with the iron. The average r.m.s. fluctuation of the 56 heavy atoms in this neighbourhood is 0.64 Å, compared with 0.72 Å for the other 411 heavy atoms of the protein interior. The average r.m.s. fluctuations are larger in the disk on the Met 80 side (0.71 Å) than in the disk on the His 18 side (0.56 Å).

The position fluctuations of the backbone atoms show a correlation with secondary structure (Fig. 1b). The average r.m.s. fluctuation of C α positions in the α helices is 0.65 Å, while that of the other C α atoms is 0.72 Å. Within the α helices, residues which have the smallest surface exposures tend to have the smallest fluctuations (for example, residues 6, 9, 10, 52, 64, 67, 71 and 74 in Fig. 1a,c).

The correlations of atomic mobility with structural features in cytochrome c are in general accord with the results of theoretical¹⁰⁻¹⁴ and experimental^{20,21} studies of other proteins. Relatively small fluctuations of interior atoms and of backbone atoms in regions of secondary structure were found in simulation studies of the pancreatic trypsin inhibitor¹¹⁻¹⁴ and in analyses of the X-ray temperature factors for myoglobin²⁰ and lysozyme²¹. The myoglobin study²⁰ also revealed an asymmetrical reduction in the fluctuations of protein atoms on the two sides of the haem group.

The dynamical simulation described here is being repeated with more rigorous equilibration. The magnitudes of the fluctuations will apparently be somewhat smaller, but the structural correlations will be unchanged. Detailed accounts of this simulation and of a planned simulation of the oxidized form will be presented in the future.

This work was supported in part by the NSF and by the Petroleum Research Fund, administered by the American Chemical Society. S.H.N. is a Robert A. Welch Foundation Postdoctoral Fellow.

Received 4 February; accepted 16 April 1980.

- Dickerson, R. E. & Timkovich, R. in *The Enzymes*, 11, 3rd edn, Ch. 7 (Academic, New York, 1975).
- Salemme, F. R. *A. Rev. Biochem.* **46**, 299-329 (1977).
- Mandel, N. *et al. J. biol. Chem.* **252**, 4619-4636 (1977).
- Labhardt, A. & Yuen, C. *Nature* **277**, 150-151 (1979).
- Campbell, I. D. *et al. FEBS Lett.* **70**, 96-100 (1976).
- Cookson, D. J. *et al. Eur. J. Biochem.* **83**, 261-275 (1978).
- Ulmer, D. D. & Kägi, J. H. R. *Biochemistry* **7**, 2710-2717 (1968).
- Patel, D. J. & Canuel, L. L. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1398-1402 (1976).
- Hopfield, J. J. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3640-3644 (1974).
- McCammon, J. A., Wolyne, P. G. & Karplus, M. *Biochemistry* **18**, 927-942 (1979).
- Karplus, M. & McCammon, J. A. *CRC Cr. Rev. Biochem.* (in the press).
- McCammon, J. A. & Karplus, M. *A. Rev. phys. Chem.* (in the press).
- McCammon, J. A., Gelin, B. R. & Karplus, M. *Nature* **267**, 585-590 (1977).
- Karplus, M. & McCammon, J. A. *Nature* **277**, 578 (1979).
- Linder, B. *Adv. chem. Phys.* **12**, 225-282 (1967).
- Takano, T. *et al. J. biol. Chem.* **252**, 776-785 (1977).
- Bernstein, F. C. *et al. J. molec. Biol.* **112**, 535-542 (1977).
- Levitt, M. *J. molec. Biol.* **82**, 393-420 (1974).
- Richards, F. M. *A. Rev. Biophys. Bioengng* **6**, 151-176 (1977).
- Frauenfelder, H., Petsko, G. A. & Tserrnoglou, D. *Nature* **280**, 558-563 (1979).
- Artymiuk, P. J. *et al. Nature* **280**, 563-568 (1979).

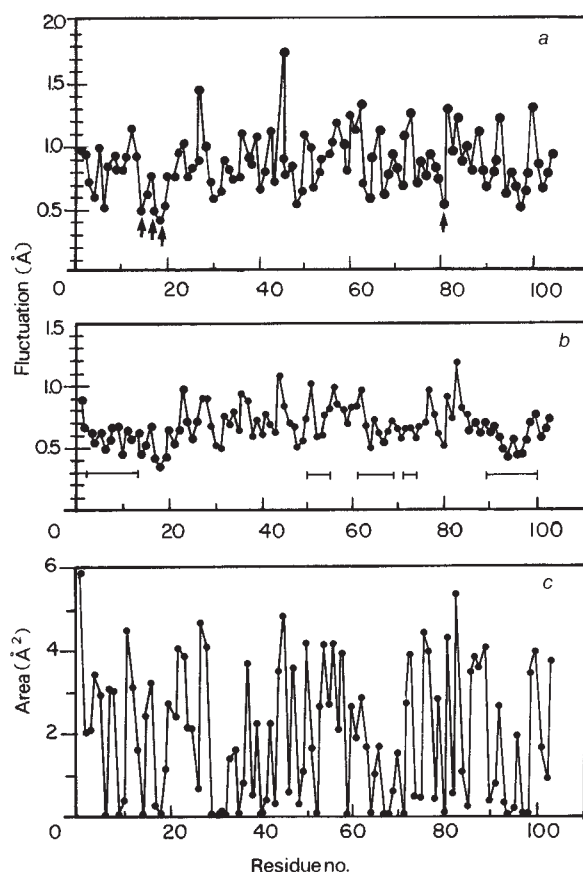


Fig. 1 *a*, Average r.m.s. atomic position fluctuation for each residue. Arrows indicate the residues (14, 17, 18 and 80) that are covalently bonded to the haem group. *b*, r.m.s. position fluctuations of the C α atoms. Bars are drawn to indicate the α -helical segments. *c*, Exposed surface area of each residue in the dynamical average structure, divided by the number of heavy atoms in the residue. The exposed surface area is the accessible contact surface area as calculated by Richards's method¹⁹ using a probe of 1.4-Å radius.

MATTERS ARISING

Cometary evidence against the solar companion

WILKINS¹ has concluded, from his computation of the perturbation of cometary orbits, that the Sun cannot have a fast moving neutron star as a companion. His results exclude all objects other than a massive black hole ($350 M_{\odot}$) with velocity $\gtrsim 10^2 \text{ km s}^{-1}$ as candidates for the solar companion whose existence was proposed by Harrison². In a previous paper³ I performed a closely similar calculation and concluded that an object of solar mass cannot be ruled out as a solar companion on the basis of cometary evidence, provided that its speed exceeds 20 km s^{-1} . Our results are thus in conflict by several orders of magnitude.

In equation (8) of Wilkins' paper, the perturbation of a comet's energy is given, to an order of magnitude, by the difference in the potential exerted by the companion at the end points of the comet's orbit, that is

$$\Delta E \sim \frac{Gm_c}{D} \cdot \frac{r_{\odot i} \cos \theta}{D}.$$

If m_c and D are now increased, such that the ratio m_c/D^2 remains constant, the resulting perturbation is unchanged. This means that the perturbation as calculated by Wilkins would still exist if the Sun and comet were freely falling in a uniform gravitational field; the calculation must therefore be in error.

A correct evaluation of the perturbation of the cometary orbit uses the gradient of the disturbing function due to the companion⁴, as in equation (9) of ref. 3. In this case the perturbation

$$\Delta\left(\frac{1}{a}\right) \propto Gm_c/D^2 v$$

goes to zero as D becomes large, since the velocity v scales with D .

J. G. KIRK

Max-Planck-Institut für Physik & Astrophysik,
Institut für Astrophysik,
Karl-Schwarzschild-Strasse 1, 8046
Garching bei München, FRG

WILKINS REPLIES The black hole was mentioned as only one example satisfying the luminosity criteria of Pineault¹. But its supposed distance (15,000 AU) and mass may need to be increased severalfold.

Note that Kirk uses a different reference frame to calculate ε , the specific energy (per unit mass) of the comet (K). Kirk finds ε_i relative to the instantaneous rest frame of the Sun (S), which is accelerated by the companion (C). I, however, determine ε_F relative to a fixed inertial frame, that of the Sun at apparition. These energies increase (Δ) as follows:

$$\Delta \varepsilon_i = \int (\mathbf{f}_{CK} - \mathbf{f}_{CS}) \cdot (\mathbf{v}_K - \mathbf{v}_S) dt \quad (1)$$

$$\Delta \varepsilon_F = \int \mathbf{f}_{CK} \cdot \mathbf{v}_K dt - \int \mathbf{f}_{KS} \cdot \mathbf{v}_S dt \quad (2)$$

(In general ε_i is the total energy of the binary system K-S referred to its centre of mass, divided by its reduced mass.) Here $\mathbf{f}_{ij}$ is the force (per unit comet mass) of body i on j . Equation (1) is the integral form of Kirk's equation (9), divested of esoteric notation. Integration by parts of equation (2) yields $\Delta \varepsilon_F = \Delta[\varepsilon_i + \mathbf{v}_S \cdot (\mathbf{v}_K - \mathbf{v}_S) + v_S^2/2]$, which also follows from the definitions of $\varepsilon_{F,i}$. Because the cross-term vanishes for a parabolic comet and because the last term $\simeq (Gm_c/Rv_C)^2$, where $R = r_{CS}$ at apparition, is small, $\Delta \varepsilon_F \simeq \Delta \varepsilon_i$.

The discrepancy between my results and those of Kirk arose from my too-crude approximation for the first integral of equation (2), and the second integral should have been three times larger than in my equation (6). Thus I did not realize that the two integrals would cancel almost completely, leaving a difference smaller than either by about r_i/R (defined after equation (3)).

A head-on approach of C towards S, at high speed $v_C = \text{constant}$, can be calculated analytically. Introducing the semi-major axis a through $1/a = -2\varepsilon_i/Gm_S$, one finds

$$(1/a) = -0.22(3 \cos^2 \psi - 1) \times (m_C/m_S)[(r_i/R)^2 + 0(r_i^3/R^3)]R^{-1} \quad (3)$$

ψ is the polar angle from axis CS about S, of the comet's zero-energy radial infall to the Sun; the comet's distance from the Sun at a time $2R/v_C$ before apparition is denoted by $r_i = (9Gm_S/2)^{1/3}(2R/v_C)^{2/3}$. Averaging over the isotropic flux of comets, one finds that the mean value of $1/a$ is unchanged, but its r.m.s. spread due to C, equals $\sigma_C = 0.2 \times (m_C/m_S)(r_i/R)^2 R^{-1}$. Demanding that $\sigma_C < 2 \times 10^{-5} \text{ AU}^{-1}$ (as for comets

without non-gravitational forces²) and setting $g_C = Gm_C/R^2$ yields the constraint

$$(g_C/10^{-6} \text{ cm s}^{-2})^{1/3} (R/10^3 \text{ AU})^{1/3} \times (30 \text{ km s}^{-1}/v_C)^2 < 0.86 \quad (4)$$

If, for example, the first two factors are each unity, this gives $v_C > 32 \text{ km s}^{-1}$, exceeding Kirk's estimate. Actually, the lower limit should be still larger because an off-side collision prolongs the epoch of closest approach and unless v_C were augmented, σ_C would increase.

The smallness of Neptune's perturbations, however, forbids a close encounter unless the companion has speeds well above 10^2 km s^{-1} . Let $T = 2R/v_C$ be the effective transit time (10^2 yr for $R = 10^3 \text{ AU}$, $v_C = 10^2 \text{ km s}^{-1}$). Neptune's known perturbations roughly limit

$$(Gm_C/R^3) \times \begin{cases} T > 170 \text{ yr} & (a) \\ (T/170 \text{ yr})^2, T < 170 \text{ yr} & (b) \end{cases} \quad (5)$$

to less³ than $1.2 \times 10^{-24} \text{ s}^{-2}$. Case (a) yields

$$R > 5.3 \times 10^4 \text{ AU} (g_C/10^{-6} \text{ cm s}^{-2}) \quad (6)$$

This excludes a close approach lasting more than 170 yr. Only a briefer encounter, case (b), permits $R \sim 10^3 \text{ AU}$, if the speed is high enough:

$$v_C > 440 \text{ km s}^{-1} (R/10^3 \text{ AU}) \times (g_C/10^{-6} \text{ cm s}^{-2})^{1/3} \quad (7)$$

This easily predominates over the cometary limit above.

My article gave a limit on R three times smaller than in equation (6) because I had adopted the allowed tidal gradient⁴ for a companion in circular orbit at $r_{CS} \leq 75 \text{ AU}$, which turns out unexpectedly to be three times larger than the value³ for $r_{CS} = 150 - 600 \text{ AU}$. Equations (5), (6) use the latter value, assuming that it has already stabilized at its large-distance limit.

Note added in proof: In work to be published I confirm that the right side of equation (4) never exceeds 0.86 for any fast encounter.

DANIEL WILKINS

Istituto di Astrofisica Spaziale del CNR,
CP 67, I-00044, Frascati, Italy

1. Wilkins, D. *Nature* **282**, 696-697 (1979).

2. Harrison, E. R. *Nature* **270**, 324-326 (1977).

3. Kirk, J. G. *Nature* **274**, 667-669 (1978).

4. Smart, W. M. *Celestial Mechanics*, 9 (Longmans, London, 1953).

1. Pineault, S. *Nature* **275**, 727-730 (1978).

2. Marsden, B. G., Sekanina, Z. & Everhart, E. *Astr. J.* **83**, 64-71 (1978).

3. Rawlins, D. & Hammerton, M. *Mon. Not. R. astr. Soc.* **162**, 261-270 (1973).

4. Rawlins, D. & Hammerton, M. *Nature* **240**, 457 (1972).

Redshifts and distances

IN the recent review by Burbidge¹ I had expected to find a wide ranging discussion of all the important evidence both for and against cosmological origin of quasar redshifts. However, no mention is made of how the continuity of properties between galaxies and QSOs and amongst QSOs themselves supports the cosmological interpretation of all QSO redshifts, or how the number/redshift distribution of bright QSOs is found to be incompatible with non-cosmological redshifts². Even within his rather restricted brief (dealing only with QSO/galaxy associations) Burbidge has failed to include results which do not favour his conclusions. He cites the existence of five 3C QSOs near bright galaxies as evidence for non-cosmological redshifts³. However, he does not mention several subsequent investigations⁴⁻⁶ of much larger samples of QSOs which failed to detect a statistically significant excess of close pairs compared with that expected by chance for a random distribution of quasars and galaxies.

Burbidge ignores published criticism of at least one of the arguments he uses. His Fig. 1 shows a correlation between galaxy redshift and separation of the galaxy from the associated QSO, and this is taken as good evidence for the galaxies and the QSOs being physically associated. Nevertheless, it has been pointed out by Noerdlinger⁷ and Browne and Cohen⁵ that when pairs selected from heterogeneous samples are used, strong selection effects inevitably give rise to a redshift-separation correlation roughly of the form presented by Burbidge. Note that not only is the legend to Fig. 1 confusing because it refers to a symbol not present in the diagram, but also the line shown is certainly not the best fit to all the points.

Finally, to establish the reality of non-cosmological redshifts systematic investigations of well defined samples must be undertaken. Then, at least, it will be unnecessary to resort to estimating Arp's allocation of telescope time as a way of assessing the statistical significance of the results.

IAN W. A. BROWNE

Moss Cottage, Strawberry Lane,
Wilmslow, Cheshire SK9 6AH, UK

1. Burbidge, G. R. *Nature* **282**, 451 (1979).
2. Green, R. F. & Schmidt, M. *Astrophys. J. Lett.* **220**, L1 (1978).
3. Burbidge, E. M., Burbidge, G. R., Solomon, P. M. & Strittmatter, P. A. *Astrophys. J.* **170**, 233 (1971).
4. Hazard, C. & Sanitt, N. *Astrophys. Lett.* **11**, 77 (1972).
5. Browne, I. W. A. & Cohen, A. M. *Mon. Not. R. astr. Soc.* **182**, 181 (1978).
6. Nieto, J.-L. *Astr. Astrophys.* **70**, 219 (1978).
7. Noerdlinger, P. D. *Astrophys. Space Sci.* **38**, 457 (1975).

BURBIDGE REPLIES—Browne is clearly strongly biased in favour of the cosmological redshift hypothesis, because none of his criticisms considers that I omitted discussion of the many problems associated with that hypothesis. His remarks imply that I have omitted only work critical of the evidence for non-cosmological redshifts. In fact I attempted to demonstrate that there is good evidence for the existence of both cosmological and non-cosmological redshifts. I spent more time discussing the Arp evidence to attempt to redress the balance as the normal practice is to quote Stockton and ignore Arp.

In a previous review article¹, which was far more wide-ranging, I did discuss all of the earlier evidence for association between galaxies and QSOs. In my later article I only quoted the 3C survey evidence for historical reasons, as I was concerned with an account of the more recent work. I had overlooked the paper of Browne and Cohen² which should certainly be taken into account in evaluating my Fig. 1.

The results of Green and Schmidt³ lay outside my restricted brief. Had I discussed their results I would also have had to discuss other evidence, such as the existence of peaks and periodicities in the redshift distribution, the Compton problem, the Hubble diagram for QSOs, and the existence of so-called superlight velocities, all of which have to be discussed and evaluated before a balanced picture can be obtained.

GEOFFREY BURBIDGE

Kitt Peak National Observatory,
950 North Cherry Avenue,
PO Box 26732,
Tucson, Arizona 85726

1. Burbidge, G. *Nature phys. Sci.* **246**, 17 (1973).
2. Browne, I. W. A. & Cohen, A. M. *Mon. Not. R. astr. Soc.* **182**, 181 (1978).
3. Green, R. F. & Schmidt, M. *Astrophys. J. Lett.* **220**, L1 (1978).

The Lizard complex as an ophiolite

KIRBY¹ has stated "Of prime importance to the proposed ophiolitic nature of the complex is the relationship between peridotite and gabbro... Green's lithological map and the author's field observations show that the gabbro is further restricted to the primary-type peridotite and does not occur in the recrystallized varieties except as isolated and deformed scraps".

This is an oversimplification: Green² refers to the mineralogical assemblage within the primary peridotite and not to a completely unrecrystallized condition.

He wrote that the "coarse anhedral texture with curving grain boundaries" was igneous but concluded that "the primary peridotite assemblage has a... texture compatible with deep seated igneous crystallization or at least of crystallization in a hydrostatic or near hydrostatic stress environment. Although the banding and incipient augen foliation are ascribed to crystalline flow of the body at a very early stage, this flow has occurred at a rate accommodated in the rock without cataclasis or creation of granulitic or schistose fabrics".

All the evidence of Flett³, Green⁴ and Rothstein^{5,6} taken together suggests that the rocks within the mass of primary peridotite south of Coverack have undergone a near solid-state deformation, although within this primary assemblage peridotite, in the lherzolites and harzburgites containing little or no troctolite, there are relics of primary textures and structures resembling crescumulates rather than settled gravity stratified cumulates. The deformation of the primary assemblage structures in this area is part of the continuity which exists between the two major areas of peridotite with respect to the trends of the near vertical foliation and banding, the primary mineralogy (to be reported elsewhere), and the rare earth content⁷. It would seem reasonable, therefore, to regard the peridotite as originally one body (albeit the eastern part not quite so heavily deformed as the western part). This being so the continuity extends between the two parts of the peridotite and the hornblende schists, and is broken, one way or the other, by the cross-cutting field relationships of the gabbro. This implies a contrary view to that expressed by Kirby¹ "that the association of peridotite and gabbro existed before their juxtapositioning with the surrounding hornblende schists (the event in which some of the peridotite was recrystallized)". This does not preclude the possibility of a deformed and recrystallized eastern part of the peridotite undergoing some partial melting.

A. T. V. ROTHSTEIN

Department of Geology,
Portsmouth Polytechnic,
Portsmouth, Hampshire PO1 3QL, UK

1. Kirby, G. A. *Nature* **282**, 58-61 (1979).
2. Green, D. H. J. *Petrol.* **5**, 134-188 (1964).
3. Flett, J. S. & Hill, J. B. *Mem. geol. Surv. U.K.* (1912, 1946).
4. Green, D. H. J. *Geol.* **72**, 543-563 (1964).
5. Rothstein, A. T. V. *Geol. Mag.* **108**, 393-398 (1971).
6. Rothstein, A. T. V. *Proc. geol. Ass.* **88**, 93-105 (1977).
7. Frey, F. A. *Geochim. cosmochim. Acta* **33**, 1429-1447 (1969).

KIRBY REPLIES—The interrelationships between the foliations in the primary peridotite and those in the secondary peridotites and schists are not well

exposed anywhere. However, on the south-east coast of the Lizard peninsular the foliation and banding in the primary peridotite, although mostly poorly defined, dips at moderate angles. The foliation in the underlying hornblende schists is subhorizontal. Green¹ described an increase in the metamorphic grade of the schists in approaching the overlying peridotite, demonstrating that the peridotite was the local heat source. The non-parallelism of the foliations implies that there is not a continuum in deformations as proposed by Rothstein. It shows that the development of foliations in the schists is a later event. In the absence of a continuum of deformation, the cross-cutting relationships of gabbro in primary peridotite do not imply that the gabbro was intruded after the development of the hornblende schists and secondary peridotites. Gabbro is very rare in the secondary peridotites and where it does occur it is invariably highly deformed. I have seen no gabbro intrusive into hornblende schists. These admittedly simplified observations suggest that the gabbro was intruded into the primary peridotite before the deformational event which led to the formation of the hornblende schists and secondary peridotites, and further that it was preferentially intruded into parts of the primary peridotite that generally escaped later deformation. These relationships are typical of ophiolite complexes in which gabbro intrudes peridotite with mantle-tectonite fabrics towards the top of the complex whereas at the base, this peridotite contains little or no gabbro and is overprinted by a later deformation associated with the formation of hornblende schists, as summarized recently by Nicholas *et al.*³.

G. A. KIRBY

Deep Geology Unit,
Institute of Geological Sciences,
Keyworth, Nottingham NG12 5GG, UK

1. Green, D. H. *J. Geol.* **72**, 543-563 (1964).

2. Nicholas, A., Boudier, F. & Boudier, J. L. *Am. J. Sci.* (in the press).

Evolution of bacterial cytochromes c_2

IN an attempt to uncover the phylogeny of cytochromes c_2 , Ambler *et al.*¹ recently compared 13 cytochrome c_2 primary structures. The molecules they compared were from purple photosynthetic bacteria and could be classified on the basis of the length of their interhelical loops as belonging to three different cytochrome c_2 subclasses² (so-called short, medium and long cytochromes c_2). The molecular phylogeny reported by Ambler *et al.* is at apparent variance with a morphological

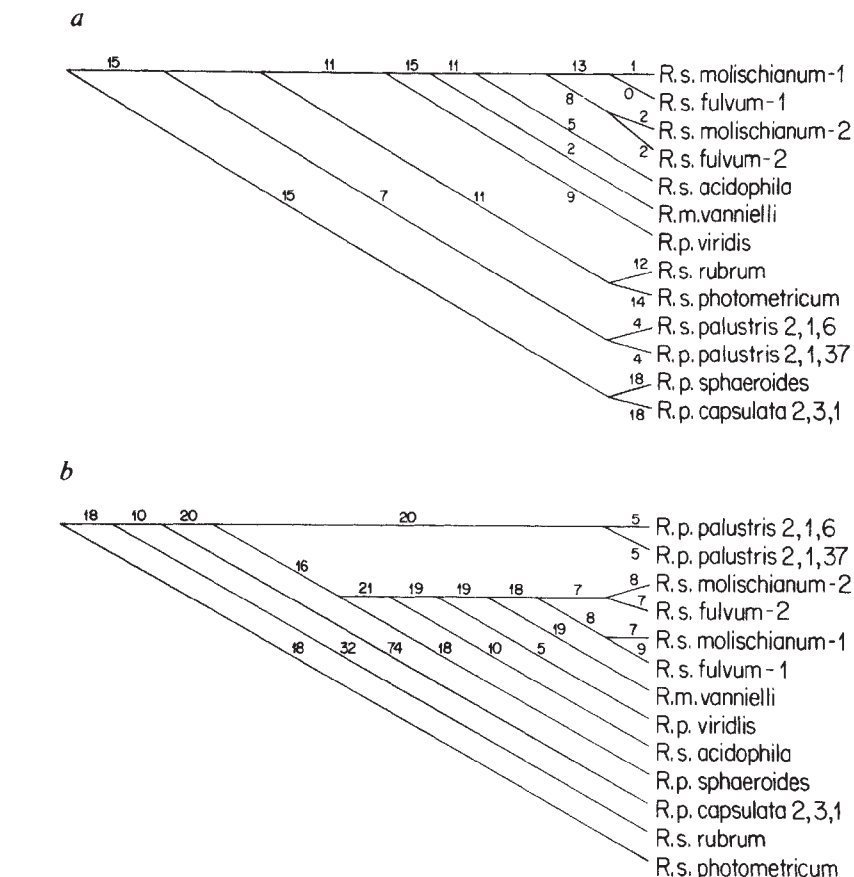


Fig. 1 Phylogenetic trees⁷ of bacterial cytochromes c_2 . For abbreviations see Table 1. *a*, Tree constructed using the similarity matrix of the unstructured segments. *b*, Tree constructed using the similarity matrix of the secondary structure segments. Numbers give branch lengths in arbitrary units.

one³ and we should like to comment on this challenging fact.

The phylogeny of Ambler *et al.* is based on a per cent similarity matrix of the whole primary structures (a matrix element represents % of identical amino acid residues found in a pair of primary structures aligned to maximize homology). It has been shown previously^{4,5} that different segments of the primary structure which correspond to individual secondary structure elements (helices, pleated sheets, turns)

evolve at a different rate (that is, they display different percentages of similarity) according to the different functional constraints imposed on them. We have therefore constructed two similarity matrices of the 13 cytochrome c_2 structures: the first contains per cent similarities of those primary structure segments which are involved in the secondary structures, the second consists of per cent similarities displayed by unstructured segments. (Assignment of the segments to the secondary structure

Table 1 Similarity matrix of bacterial cytochromes c_2

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. <i>Rm. vannielli</i>	100	59	57	51	46	52	55	38	38	42	41	35	28
2. <i>Rp. viridis</i>	65	100	52	43	43	43	45	33	32	42	41	32	28
3. <i>Rs. acidophila</i>	74	79	100	38	33	38	41	35	35	32	35	45	28
4. <i>Rs. molischianum-1</i>	68	60	69	100	84	70	75	30	36	35	35	29	26
5. <i>Rs. fulvum-1</i>	69	62	71	99	100	72	70	30	33	32	33	28	28
6. <i>Rs. molischianum-2</i>	76	69	75	78	79	100	86	30	36	35	35	25	26
7. <i>Rs. fulvum-2</i>	76	68	74	76	78	96	100	29	38	32	32	29	26
8. <i>Rs. rubrum</i>	54	51	54	56	57	62	60	100	54	41	39	41	38
9. <i>Rs. photometricum</i>	51	53	51	57	56	60	60	74	100	39	33	33	32
10. <i>Rp. palustris 2,1,6</i>	51	47	51	49	49	59	59	53	51	100	90	39	45
11. <i>Rp. palustris 2,1,37</i>	51	47	51	49	49	59	59	53	51	91	100	42	41
12. <i>Rp. sphaeroides</i>	35	35	37	34	35	41	43	49	47	32	32	100	43
13. <i>Rp. capsulata 2,3,1</i>	40	43	40	46	47	50	49	59	53	41	41	63	100

Numbers represent per cent identical amino acid residues in a pair of the primary structure segments compared. Above the diagonal (upper right): numbers obtained for segments with secondary structure; below the diagonal (lower left): numbers obtained for unstructured segments. Abbreviations *Rs.*, *Rhodospirillum*; *Rp.*, *Rhodopseudomonas*; *Rm.*, *Rhodomicrobium*.

elements—four α -helices, two 3_{10} helices and three turns—is based on the three-dimensional structure of *Rhodospirillum rubrum* cytochrome c_2 (ref. 6) deposited with the Brookhaven Protein Data Bank.) The two matrices computed in this way clearly differ from one another (see Table 1). When phylogenetic trees⁷ are constructed of them (Fig. 1), these also differ.

There are 135 amino acid positions in the cytochromes c_2 , half of which (69) form helices and turns, the other half (66) being involved in unstructured loops. There is therefore no bias in favour of any of the two groups considered. They do differ evolutionarily, however, in that the secondary structure segments predominantly evolved by amino acid replacements whereas the unstructured loops harbour two-thirds of the deletions responsible for a distinction of the short, medium and long cytochromes c_2 .

Thus, the following important points emerge as far as evolution of cytochromes c_2 is concerned. (1) Different phylogenies can be proposed for different segments of homologous primary structures, the phylogeny of the whole structure being a weighted average of the partial ones. (2) Because the differences can be correlated with typical features of the secondary structure, they seem to result from structural, rather than genetic causes.

Bacterial phylogeny is a notoriously difficult subject. Dickerson⁸ and Woese *et al.*⁹ have already tried to reconcile the cytochrome c_2 dilemma; this note, far from attempting to solve all the existing questions, aims at delineating a new approach to them.

We thank Dr Edgar Haber for discussions and for facilities used in the preparation of this manuscript.

JIRÍ NOVOTNÝ

Molecular and Cellular
Research Laboratory,
Massachusetts General Hospital,
Harvard Medical School
Boston, Massachusetts 02114

ANTONÍN VÍTEK

Institute of Organic Chemistry
and Biochemistry,
Czechoslovak Academy of Sciences,
166 10 Prague 6,
Czechoslovakia

6. Salemme, F. R., Freer, S. T., Xuong, N. H., Alden, R. A. & Kraut, J. *J. biol. Chem.* **248**, 3910–3921 (1973).
7. Fitch, F. W. & Margoliash, E. *Science* **155**, 279–284 (1967).
8. Dickerson, R. E. *Nature* **283**, 210–212 (1980).
9. Woese, C. R., Gibson, J. & Fox, G. E. *Nature* **283**, 212–214 (1980).

Methotrexate binding to dihydrofolate reductase

RECENTLY, in discussing dihydrofolate reductase, Gready¹ described, with the emphasis on our paper², the finding that the inhibitor, methotrexate, binds to the enzyme with its pteridine ring 'upside down' with respect to the substrates dihydrofolate and folate. It is essential to point out that the most important observations leading to this conclusion were made by others.

In their report of the crystal structure of the enzyme-methotrexate-NADPH complex, Matthews *et al.*³ discussed the implications for the stereochemistry of the reaction and specifically recognized the possibility that the substrates might bind the other way up. Shortly thereafter, Fontecilla-Camps *et al.*⁴ determined the absolute configuration of 5,10-methenyl-tetrahydrofolate (which can be related to that of tetrahydrofolate itself; see ref. 2). This revealed the stereochemistry of reduction of dihydrofolate at C6, and by comparison with the results of Matthews *et al.*, showed that dihydrofolate does bind the other way up from methotrexate. This was pointed out by Hitchings⁵. Our NMR experiments extended this to folate, showing that both substrates have the same orientation on the enzyme, which, from the crystallographic work, differs from that adopted by methotrexate.

The interesting finding that substrates and structurally analogous inhibitors bind to dihydrofolate reductase in quite different orientations thus arises from a comparison of a number of separate observations.

G. C. K. ROBERTS
J. FEENEY
B. BIRDSALL

National Institute for Medical Research,
The Ridgeway, Mill Hill,
London NW7 1AA, UK

P. CHARLTON
D. YOUNG

University of Sussex,
Falmer, Brighton,
Sussex BN1 9RH, UK

Effects of the Earth's rotation rate on climate

HUNT'S¹ investigation of the meteorological effects of a faster rotating Earth has application to palaeoclimatology, but his findings cannot explain the occurrence or character of the several late Precambrian glaciations.

Hunt's¹ article does not distinguish between middle and late Precambrian. Late Precambrian glaciation occurred $0.95\text{--}0.60 \times 10^9$ yr ago^{3–5}, whereas glaciation is unknown during the middle Precambrian $\sim 1.5 \times 10^9$ yr ago³. Pannella's² curve of length of year suggests a 19–20-h day during the late Precambrian. Using Hunt's¹ Fig. 4, an Equator-to-Pole temperature difference of ~ 41 K and poles ~ 4 K colder than at present accordingly are indicated for late Precambrian glacial time; these estimations assume an obliquity of the ecliptic near that of today, which geological^{3–7} and geophysical^{3,5,8} data suggest may be invalid.

In the Precambrian conditions predicted by Hunt's¹ model—an increased Equator-to-Pole temperature gradient, with equatorial regions warmer and polar regions colder than those of today—glaciation would be confined to high palaeolatitudes, while low palaeolatitudes remained ice-free. Available palaeomagnetic data^{3,5,8} reveal, however, a dominant picture of low palaeolatitude ($\sim 0\text{--}40^\circ$) glaciation in late Precambrian time; although some of the data are difficult to interpret⁹, circumpolar models of glaciation can be ruled out. Furthermore, Hunt's¹ findings cannot explain the marked episodicity of the seven or more glaciations since the middle Precambrian^{3,4}.

The major application of Hunt's¹ model lies in testing explanations of low-palaeolatitude glaciation in the late Precambrian. The concept of global glaciation^{3,8} as a cause of preferred low-palaeolatitude glaciation is difficult to reconcile with an Earth possessing a warmer Equator and an Equator-to-Pole temperature gradient greater than at present. The alternative hypothesis of an increased obliquity of the ecliptic ($\geq 54^\circ$) in late Precambrian time^{3–8} to reduce equatorial insolation and thereby confine glaciation to relatively low palaeolatitudes explains the data better.

G. E. WILLIAMS

BHP Melbourne Research Laboratories,
Clayton, Victoria 3168,
Australia

1. Hunt, B. G. *Nature* **281**, 188–191 (1979).
2. Pannella, G. *Astrophys. Space Sci.* **16**, 212–237 (1972).

1. Ambler, R. P., Meyer, T. E. & Kamen, M. D. *Nature* **278**, 661–662 (1979).
2. Salemme, F. R. *Rev. Biochem.* **46**, 299–329 (1977).
3. Buchanan, R. E. & Gibbons, N. E. (eds.). *Bergey's Manual of Determinative Bacteriology* 8th edn (Williams & Wilkins, Baltimore, 1974).
4. Novotný, J. & Franěk, F. *Nature* **258**, 641–643 (1975).
5. Novotný, J., Vítek, A. & Franěk, F. *J. molec. Biol.* **113**, 711–718 (1977).

1. Gready, J. E. *Nature* **282**, 674 (1979).
2. Charlton, P. *et al. Chem. Commun.* 922 (1979).
3. Matthews, D. *et al. J. biol. Chem.* **253**, 6946 (1978).
4. Fontecilla-Camps, J. *et al. in Chemistry and Biology of Pteridines* (eds Kisluk, R. L. & Brown, G. M.) 235 (Elsevier, Amsterdam, 1979).
5. Hitchings, G. H. *Enzyme Inhibitors as Drugs* (ed. Sandler, M.) (Macmillan, Basingstoke, 1980).

3. Frakes, L. A. *Climates Throughout Geologic Time* (Elsevier, Amsterdam, 1979).
4. Williams, G. E. *Megacycles: Long-Term Episodicity in Earth and Planetary History* (Dowden, Hutchinson & Ross, Stroudsburg, in the press).
5. Williams, G. E. *Geol. Mag.* **112**, 441–465 (1975).
6. Young, G. M. *Am. J. Sci.* **276**, 366–370 (1976); *Precambrian Res.* **3**, 137–158 (1976).
7. Kröner, A. *J. Geol.* **85**, 289–300 (1977).
8. McWilliams, M. O. (thesis, Australian National Univ. (1977); *Trans. Am. geophys. Un.* **59**, 1061 (1978).
9. Morris, W. A. *Geology* **5**, 85–88 (1977).

HUNT REPLIES—Current knowledge regarding climatic conditions during the Precambrian is fragmentary and subject to differing interpretations. However, there is considerably more information available than I was aware of, mainly in the geological literature, and I thank Dr Williams for providing very interesting data. Frakes¹ suggests that glacial conditions prevailed 2,300 Myr ago, followed by a warm period extending to 950 Myr when an interval of episodic ice ages commenced, terminating at about 600 Myr (ref. 2). If, as I suggested³, 1,500 Myr ago the Earth was rotating 2–2.5 times faster than now with a somewhat smaller obliquity, climatic conditions should have been most conducive to an ice age. Thus the warm period which apparently then existed implies some very marked difference between the model and the actual atmosphere. What this difference was remains a mystery! With regard to the late Precambrian (600–1,000 Myr ago) when the Earth's rotation was closer to its present rate, Williams² suggests that glaciation was confined to low and middle latitudes, attributable to the Earth's obliquity then exceeding 54°. Clearly any such obliquity variations would create climatic conditions completely different from those of my model regardless of rotation rate effects.

The concept of climatic changes induced by obliquity variations has considerable attractions, but also unresolved problems. Williams² has suggested an obliquity cycle with a period of over 1,000 Myr and a total amplitude exceeding 54°. Wolfe⁴, from Tertiary palaeobotanical data, has deduced obliquity variations of up to 25° in ~25 Myr. No mechanism is advanced to account for these obliquity changes. The absence of an identifiable mechanism, however, should not preclude them from consideration; rather it should stimulate a search for the cause. If such obliquity changes did occur this implies that current understanding of the Earth–Moon dynamical interaction over the ages as presented by MacDonald⁵, for example, is deficient. The rotational period and obliquity variations with time shown in my Fig. 1 (ref. 3) were taken from MacDonald's⁵ calculations, and had the great attraction of being dynamically based and mutually consistent.

One positive way to investigate the hypothesis of obliquity-induced climatic

variations is to resort to numerical modelling; as noted by Williams this approach has considerable potential. In 1980 I intend to run the same model as used in my study³, with an obliquity of 0° and a value near 60° as an initial contribution to this fascinating area of science. It is hoped this will throw light on the combined climatic effects of an increased rotation rate and extreme values of obliquity.

B. G. HUNT

Australian Numerical Meteorology Research Centre, PO Box 5089AA, Melbourne, Australia 3001

1. Frakes, L. A. *Climates Throughout Geologic Time* (Elsevier, Amsterdam, 1979).
2. Williams, G. E. *Geol. Mag.* **112**, 441–465 (1975).
3. Hunt, B. G. *Nature* **281**, 188–191 (1979).
4. Wolfe, J. A. *Am. Scient.* **66**, 694–703 (1978).
5. MacDonald, G. J. F. *Rev. Geophys.* **2**, 467–540 (1964).

Analysis of host specificity of temperate and tropical animals

IN the contingency-table analysis of the host specificity of temperate and tropical ambrosia beetles, Beaver¹ erred in using the percentages of species showing various grades of host specificity rather than the actual frequencies². Depending on the sample size and the distribution of frequencies among cells, this procedure may either inflate or deflate the values of χ^2 and thus invalidate a test of independence of rows and columns. Also, Beaver¹ implied that a test of host specificity for xylomycetophagous beetles was carried out with a 2×4 table but no xylomycetophagous species was restricted to a single species of host plant. Thus, the data for this group should have been tested in a 2×3 design.

Corrected values of χ^2 for Beaver's¹ data are given in Table 1. Considering all species of both families of beetle, the evidence for increasing specialization with increasing latitude is considerably

stronger than Beaver's analysis indicated. For phloeophagous species, the evidence is weaker than Beaver suggested but, for the most part, his hypothesis is strongly supported by data from this group of species. However, the data for the xylomycetophagous species are equivocal (4/6 country pairs show independence of rows and columns) and thus provide no evidence in support of Beaver's hypothesis. It is not clear whether this failure results from the small sample sizes for this group or from a fundamentally different pattern of resource utilization.

STEPHEN SMITH

*Faculty of Science,
Department of Biology,
University of Waterloo,
Waterloo, Ontario, Canada N2L 3G1*

1. Beaver, R. A. *Nature* **281**, 139–141 (1979).
2. Zar, J. H. *Biostatistical Analysis* (Prentice-Hall, Englewood Cliffs, 1974).

BEAVER REPLIES—The point concerning the use of percentages in χ^2 tables is accepted. I had not intended to suggest that the xylomycetophagous species followed the pattern of the phloeophagous species and were less host specific in the tropics. As I noted¹, they show “a similar low specificity in both temperate and tropical regions”. I think it probable that this difference from the phloeophagous species is related to the different methods of resource utilization. If the ambrosia fungi can grow in the dying or dead wood of a high proportion of the tree species present in a tropical forest, this forest may not appear more heterogeneous to an ambrosia beetle than a temperate forest.

R. A. BEAVER

*Department of Biology,
The University of Zambia,
PO Box 2379,
Lusaka, Zambia*

1. Beaver, R. A. *Nature* **281**, 139–141 (1979).

Table 1 Comparison* of the degree of specialization of feeding habits of Scolytidae and Platypodidae

Species group	Countries compared					
	Fr/Ca	Fr/Ma	Fr/Fi	Ca/Ma	Ca/Fi	Ma/Fi
All	15.085	165.121	70.169	245.781	100.747	20.505
Phloeophagous	13.058	37.753	40.718	55.899	40.905	12.110
Xylomycetophagous	1.640	19.339	2.148	9.583	4.161	5.126

*The null hypothesis states that there is no relationship between the degree of specialization and latitude. Data were tested in 2×4 (all species and phloeophagous species) or 2×3 (xylomycetophagous species) contingency tables. For 2×4 tables, critical values are 7.815, 11.345 and 16.266 for $\alpha = 0.05$, 0.01 and 0.001, respectively; corresponding critical values for 2×3 tables are 5.991, 9.210 and 13.816. The values of χ^2 comparisons among all pairs of countries are given. Ca, California; Fi, Fiji; Fr, France; Ma, West Malaysia.

BOOK REVIEWS

Peripatetic physicist

C.A. Russell

THE PHYSICIST Joseph Henry (1797–1878) achieved lasting fame for his work on electromagnetism and, in particular, his anticipation (1830) of Faraday's discovery of electromagnetic induction. He became, in 1832, Professor of Natural Philosophy at Princeton, and in 1846 the first Director of the Smithsonian Institution in Washington. In his later years he was active in establishing the American Association for the Advancement of Science and the National Academy of Sciences. Today, his name is perpetuated in two main ways: by the unit of electrical inductance, the henry, and by the splendid collection of his personal papers in the archives of the Smithsonian Institution. It is this deposit which forms the nucleus of *The Papers of Joseph Henry*, currently in course of publication. Two volumes have already appeared dealing with, respectively, his years at Albany, New York, until 1832, and the first part of his Princeton period. The present book covers the years 1836–1837, subsequent volumes being planned for the remainder of his life.

For a book of this size merely to span two years may seem an excessively generous use of space, but as the editor rightly observes "the period covered was extraordinarily eventful". In the study of electricity the outlines of a coherent view of electromagnetism were beginning to emerge. Developments in optics, metallurgy and engineering were bearing fruit in lighthouse construction, instrumentation, shipbuilding and the new steam railways. Thanks partly to geological progress, searching questions were being asked about the nature of science and its relation to revealed religion. And Henry's own university was struggling to equip its laboratories in a manner fitting for the new challenges, even though its efforts coincided with the great economic depression of 1837. For Henry himself an even more significant event took place at this time: a scientific tour of Europe. In his own extensive reporting of this unforgettable experience lies the greatest interest of this remarkable collection of documents.

In the first part of the book we see Henry writing to his brother James on family matters, to his wife while away on a visit to Albany and to Benjamin Silliman on

The Papers of Joseph Henry. Vol. 3: *January 1836–December 1837, The Princeton Years*. Edited by N. Reingold. Pp. 585. (Smithsonian Institution Press: Washington D.C., 1979.) \$30.

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

popular demonstrations in electromagnetism. He confides to his notebook a "record of experiments", chiefly on the lateral discharge of static electricity. Other correspondence fills out many intriguing details of the American scientific scene, and introduces us to a host of scientific figures both famous and obscure. The idea of a Continental trip was mooted in mid-1836 as a means of procuring the required scientific equipment. It was but one of many such journeys undertaken by Americans to post-Napoleonic Europe. Thereafter, foreign travel figures frequently in the correspondence until February 22 when he set sail for Europe. An increasing apprehension about the potential hazards of his voyage is evident in his letters. It would hardly have been dispelled by the request of a scientific colleague that he convey to Europe for

distribution numerous copies of his latest paper, "On the gales and hurricanes of the Western Atlantic".

The account of Henry's European tour is supplemented by one from his friend and counterpart in Philadelphia, A. D. Bache, who left for Europe a few months before Henry. Their two diaries illuminate the great sensitivity of Americans at that time to their reputation overseas. Nowhere is this more evident than in Henry's experiment in King's College, London, when he tried to obtain a spark from thermoelectricity; the successful outcome, which had eluded Faraday, gained in significance with each retelling of the tale and Henry became a folk-hero in his own country. During his London stay he frequently enjoyed the hospitality of Daniell's laboratory at King's College, and quickly became acquainted with most of the leading metropolitan scientists. His lodgings were chosen so as to be a few steps from the Royal Institution. Visits to Greenwich, Woolwich and an unnamed gasworks still further enlarged his circle of acquaintances, and he did not hesitate to characterize many of those he met. Sturgeon was reckoned to be "a very good experimenter but an indifferent lecturer"; Wheatstone was the most "talented" person he had encountered; but Faraday (who welcomed him into his own family circle and home) was "the greatest man".

From England Henry went to France where his diaries and letters reveal interesting details of transport, social life and an American's impressions of Paris. But scientifically it was less successful. Equipment ordered in France failed to be delivered, and he found considerable difficulty in meeting the great figures of French science. Partly this was due to a language problem, but also, as the editor admits, "many of Henry's opinions of France would provide ammunition for recent historiography arguing the decline of French science in the nineteenth century". Several times Henry wished he had spent less time in France and more in Britain.

The final part of his tour took him to Scotland where he gives graphic accounts of life in Edinburgh, trips to Scottish lighthouses and (especially) the Edinburgh

Observatory. Then, after attending the British Association meeting in Liverpool, and a brief visit to Cambridge, he set sail from Portsmouth on 22 October 1837 for his homeward journey.

It is impossible to do justice to the richness of material in these papers. A great number of Henry's contemporaries flit in and out of his pages: obscure instrument-makers, technicians, engineers and others

working on that period. There are eight pages of portraits (though, curiously, not one of Henry himself) and several other illustrations in the text and endpapers. For a work of this size and complexity it is

excellent value for money. Subsequent volumes will be awaited with keen interest.

C. A. Russell is Reader in History of Science at The Open University, Milton Keynes, UK.

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

on the periphery of science; relations, students, friends and casual acquaintances; politicians and royalty (though not much, for Henry was a staunch republican); poets and philosophers; and above all men and women active in science. We are treated to his views on race and slavery, on the status of women, on the cost of war, on the politics of science — and even on the philosophy of tea-making.

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

The task of the editor and his assistants has included the provision of very full notes with (where possible) full identifications of individuals, places and incidents referred to in the papers. The massive index is a testimony to their thoroughness and care. Naturally, a few blemishes have been detected; there is now some doubt about the authenticity of "Newton's" telescope at the Royal Society (p. 189); the spectrum studied by Brewster was most certainly not that of "nitrous acid" (p. 482); the Brunel referred to on p. 225 as the engineer of the Great Western Railway was not Marc but his more famous son Isambard; and there are a few uncorrected colloquialisms in the notes and a small number of misprints. But these cannot detract from the great merits of a work that will be invaluable to social historians as well as historians of science

Anecdotal astrophysics

G. T. Bath

Introduction to Advanced Astrophysics. By V. Kourganoff. Pp. 479. (Reidel: Dordrecht, The Netherlands, Boston and London, 1979.) Hardback Dfl. 135, \$71.05; paperback Dfl. 55, \$28.45.

WHEN Martin Schwarzschild wrote *Structure and Evolution of the Stars* (Princeton University Press) in 1958 (rightly referred to as "a remarkable treatise" in this book), the revolution in astrophysics which we have witnessed in recent years had already begun. For the first time computers provided a tool which led, using considerable intellectual ingenuity, to the solution of previously intractable equations describing the structure of stars and other cosmic objects. Radioastronomy had broken down barriers that restricted our view of the Universe to the optical region of the electromagnetic spectrum. New optical instrumental techniques were continuously developing and were a continuing source of unpredictable discovery.

Since then astronomy and its associated research fields have matured into a fully fledged science — one which in the UK currently receives 20% of the Science Research Council budget. The bulk of the electromagnetic spectrum is now visible through satellite or ground-based telescopes.

The new astronomies based on γ -ray, X-ray, ultraviolet and microwave observations have added a vast body of data ranging from the galactic binary X-ray sources to the cosmic microwave background. Nonetheless optical and radio wavelengths have retained their central position. In 1964 quasars were discovered, and the subject of active galaxies and galactic evolution was opened up by the mysterious optical spectra of the Cambridge 3C radio survey sources, 3C48 and 3C273. Even more startling was the way in which the persistent detective work of Bell-Burnell, Hewish and colleagues tracked down the neutron star using techniques developed to study interstellar scintillation. The Cambridge group quickly convinced the world of the reality of pulsars in 1968 (in a piece of teamwork which it does service to no one today to break down into isolated individual contributions as attempted in this book).

All this is well known. Less widely appreciated are the developments in optical instrumentation that have produced a rich source of surprises — solar oscillations, polarization and pulsations in white dwarfs, confirmation of accretion disks in stellar binary systems, complex quasar spectra reflecting structure around the quasar powerhouse and within the intergalactic medium, velocity dispersion studies providing a wealth of information on the morphology and evolution of galaxies — to mention but a few.

There is clearly a place, though competitors already exist, for a lucid, approachable and up-to-date introduction to the subject at postgraduate level: a book which reflects the past excitement, together with the logic and physics underlying models which account for these cosmic discoveries; something akin to *Structure and Evolution of the Stars*. Unfortunately, *Introduction to Advanced Astrophysics* does not provide this, at least in the way I would have hoped for.

The *Concise Oxford Dictionary of Current English* gives one definition of the word 'idiosyncrasy' as "mode of expression peculiar to an author". This book is a rich source of idiosyncrasy — of content, style, science and even English usage. In my view the author and publisher have not been successful in producing a well-balanced introduction to the study of astrophysics at the postgraduate level, which is their stated aim.

The book covers radiative transfer, stellar structure, white dwarfs, neutron stars and pulsars, binary systems and galactic X-ray sources, simple cosmological models, and, very briefly, extragalactic astronomy. The style is the lecture-note format elaborated to contain a multitude of *caveats* to simple introductory statements. These confuse the intelligent, inquiring reader, and distract from the main thrust of the subject. Most of the topics treated can hardly be described as advanced, and the treatment is muddled with anecdotes and asides.

Kepler's laws occupy 40 pages, the physics of X-ray binaries two, in a section totalling 70 pages, and the two pages on the physics are confused and misleading. Perhaps there is a place for an exhaustive discussion of the solution of binary orbital elements in a section devoted to X-ray sources, but not at the expense of a thorough discussion of the basic physical processes that power them, and make them what they are — amongst the powerful stellar energy sources in the Universe. It is a Sunday magazine supplement howler to state that in mass exchanging binaries "the

very strong gravitational field in the neighbourhood of the compact object obliges the surrounding gas to flow over to the object" from the companion (my italics).

The English is eccentric and frequently grammatically incorrect. I am full of sympathy for the problems facing scientists whose mother tongue is not English. In the preface Professor Kourganoff, in his defence, quotes Zel'dovich: "Specific broken English (not Shakespearian or slang) plays the role played by Latin in the time of Copernicus". Broken English presents no difficulty, and can even have unsuspected advantages, when reporting

original research. But textbooks are a different matter. The author has a responsibility to capture the reader's attention, to further his appreciation of the excitement of the subject, to have faith in the content and to help him use his intelligence. And that demands as much attention to style as to content. In this case I feel the author is not wholly at fault. The editor and publisher must also take some responsibility. □

G. T. Bath is a Research Fellow at Wolfson College and a Research Officer at the Department of Astrophysics, University of Oxford, UK.

Hormones from start to . . .

George Fink

Hormones and Evolution. Edited by E.J.W. Barrington. 2 vols, pp.989. (Academic: London and New York, 1979.) Each volume £30, \$69.

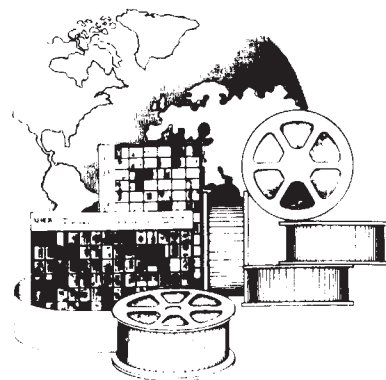
Hormones and Evolution is an important treatise which tests the hypotheses that underlie the oft-quoted aphorisms of Peter Medawar — "Endocrine evolution is not an evolution of hormones but an evolution of the uses to which they are put" — and J.B.S. Haldane — "The evolution of vertebrate hormones was nearly complete in Silurian times". Medawar's hypothesis is proved correct for the steroids which, as shown in the interesting first chapter by Sandor and Mehdi, have been found in most species of animal and many species of plant (including prokaryotes). Sandor and Mehdi argue that the steroid nucleus (cyclopentanoperhydrophenanthrene) could have been synthesized abiotically, and because steroids interact with DNA (including the DNA of bacteriophages) they could be considered to be universal biomolecules which were among the first transmitters of chemical information. Medawar's hypothesis may also hold for the monoamines and the thyroid hormones the structure, but not function, of which remains unchanged among the vertebrates.

Common to the vertebrates and many invertebrates is the fact that the nervous and endocrine systems are linked by neuroendocrine cells, the transmitter most often being a peptide. Peptide hormones have recently attracted much interest, but even so the information obtained thus far, especially about their structure in lower vertebrates and invertebrates, is too meagre to enable us to determine for most compounds whether there has been a significant evolution of peptide structure as well as function. However, in agreement with Medawar's hypothesis, the

mammalian thyrotropin releasing hormone (TRH), a tripeptide, is present in the brains of amphibians, reptiles and fish, but does not appear to release thyrotropin in these animals. Jackson suggests that TRH is a central neurotransmitter which, during evolution, acquires a role in the hypothalamic-pituitary system, first as a releasing factor for prolactin and then for thyrotropin. By contrast, the peptides of the neurohypophysis and the gut do appear to have undergone an evolution of structure as well as function. Thus, in mammals, gastrin and cholecystokinin are distinct, whereas in amphibians, fish and cyclostomes there appears to be only one gastrin/cholecystokinin molecule. In their review of the evolution of responses to the neurohypophysial principles, Sawyer and Pang point out the need in mammals for separate regulation of urine flow and milk ejection. This might explain why arginine vasopressin is the predominant pressor-antidiuretic peptide in the mammal, while arginine vasotocin predominates in non-mammalian vertebrates. Arginine vasotocin is about half as potent as oxytocin (the milk-ejection principle in mammals) in stimulating milk ejection and only half as potent as arginine vasopressin (which has little milk-ejection activity) in blocking diuresis. Thus, the switch from vasotocin to vasopressin, which only involves the substitution of phenylalanine for isoleucine (one base change in the codon), would confer a considerable advantage to the mammal. Further studies will be required to determine whether there are any common precursors for the invertebrate and vertebrate peptide hormones. Is there, for example, any relationship between the hyperglycemic hormone of the crustaceans and the gut and pancreatic hormones of the vertebrates?

As a treatise on comparative endocrinology, biologists will find this a most valuable handbook which they will wish to keep close to hand in the laboratory. Professor Barrington has chosen the topics and the authors carefully. Most vertebrate hormones and endocrine

nature



is Available in MICROFORM

For Complete Information customers should write

University Microfilms International

Dept F A
300 North Zeeb Road
Ann Arbor, MI 48106
U S A

Dept F A
18 Bedford Row
London WC1R 4EJ
England

Bring Your Library Up-To-Date With This ANS Edition...

O. J. Wick
HANDBOOK

Plutonium Handbook

A Guide to the Technology

992 pages — 8½" × 11"
Hardbound \$98.00

American Nuclear Society
555 North Kensington Avenue
La Grange Park, IL 60525 USA

Please take my order for ____ book(s).

- ☐ Payment in full enclosed.
☐ Bill me and I accept postage and handling charges.

Name _____

Organization _____

Street _____

City _____

State _____ Zip _____

systems are reviewed in detail, and one chapter each is devoted to the annelids, molluscs, crustaceans, insects and echinoderms. The chapters are mostly well written and illustrated, and reflect excellent scholarship. The authors, however, show a disappointing readiness to fit their data to prevailing views on evolution and the origin of life rather than looking for clues that might spark revolutionary ideas. There is one surprising

omission — no chapter on the pineal gland which continues to tantalize the endocrinologist and evolutionist. Perhaps in man the pineal is *only* the seat of the soul (although recent studies suggest that it may also moderate gonadal function); but what an elevation in function from a mere phototransducer! □

George Fink is Director of the MRC Brain Metabolism Unit, University Department of Pharmacology, Edinburgh, UK.

Cookery book for immunologists

A.J. Munro

Selected Methods in Cellular Immunology. Edited by Barbara B. Mishell and Stanley M. Shiigi. Pp. 486. (W.H. Freeman: San Francisco and Oxford, 1980.) \$29.95, £17.70.

THIS BOOK contains most of the principal methods used by cellular immunologists working with cells from mice. It started as a manual for teaching students and research workers in the Department of Microbiology and Immunology at the University of California, Berkeley, but has been expanded to include chapters from workers in other laboratories, although the majority of the 40 or more contributors are

from the same department. The editing and the production of the book has ensured that the style of each contribution is very similar. Each chapter starts with a brief introduction, explaining the principle behind the method to be discussed. For example, there is a clear and precise description of limiting dilution analysis before details of how to measure the frequency of precursors of B-cells, helper T-cells and cytolytic T-cells.

About half of the procedures considered are only applicable to experiments using murine cells, but the rest are of general value and make the book of interest to all immunologists. The purification of immunoglobulins and their fragments and different assay procedures for detecting the reactions of immunoglobulins are described in useful detail. There is a section devoted to the method developed by Köhler and Milstein for the production of monoclonal antibodies from hybrid cell lines which will be particularly valuable to researchers trying this procedure for the first time.

Although most of the contributions contain a list of comments, often describing particular snags in the procedure or why particular reagents have been used, this is not always the case. One feels that, as with recipes, descriptions of methods should include some indication of which steps are essential, or which reagents were used because they happened to be conveniently at hand at that moment. A further criticism concerns the expense of some of the approaches suggested — by simple modification some of the procedures could be carried out at one-tenth of the cost.

The editors have not tried to make this collection of methods comprehensive, but nevertheless they have brought together most of the important techniques for studying cellular immunology and all immunologists will find valuable procedures clearly laid out. I have had considerable difficulty in reviewing this book, since whenever I brought it into the laboratory it vanished. And, like all good cookery books, it is rapidly accumulating stains on the more used pages. □

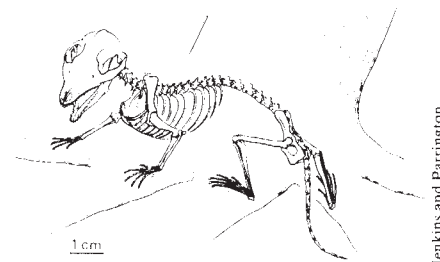
A.J. Munro works in the Division of Immunology, Department of Pathology, University of Cambridge, UK.

Mammals through the mists of time

Barry Cox

Mesozoic Mammals: The First Two-Thirds of Mammalian History. Edited by J.A. Lillegraven, Z. Kielan-Jaworowska and W.A. Clemens. Pp.321. (University of California Press: Berkeley and London, 1980.) Hardback \$30, £18; paperback \$8.75, £5.25.

THE EARLY days of mammalian evolution were perhaps the Dark Ages in more than one sense — because not only were the little early mammals probably nocturnal, but also their tiny remains are fragmentary and uncommon. It is not so long ago that it was



Skeletal reconstruction of a Triassic triconodont

said that all their known remains could have been put in a shoe-box and, though the volume of their known fossils has considerably increased, it is still true that the overwhelming majority are isolated teeth or tooth-bearing fragments of skull. Study of their evolution is therefore still largely a matter of comparative odontology, as is reflected by the names of their orders: Triconodonta, Symmetrodonta, Multituberculata etc. These Dark Ages are particularly frustrating, for they separate the period of reasonably well-known Permo-Triassic mammal-like reptile evolution, which culminated in the very mammal-like cynodonts, from the Cenozoic period of well-documented, diverse, fully-evolved marsupials and eutherians ('placentals').

This book is therefore an extremely valuable synthesis of our current knowledge of Mesozoic mammals. It includes an introduction and 14 other chapters, by a total of 12 authors — mostly from the USA, but also including Dr Zofia Kielan-Jaworowska from Poland. After the Introduction, Chapter 2 provides a summary, on a world-wide basis, of the state of the record of Mesozoic mammals, giving faunal lists for each locality or group of localities, brief discussions (where necessary) of geological age assignments and information as to what parts of the mammals are actually known from those localities. Seven of the chapters are devoted to the morphology and systematics of the different orders of Mesozoic mammal. For each of these, one table

Jenkins and Parrington

SCIENTIFIC BOOKSHOP

H. K. LEWIS can supply works in all branches of Pure and Applied Science. Catalogues on request. Please state interests.

SCIENTIFIC LENDING LIBRARY

Annual Subscription from £7.50. (Available in U.K. only)

Reduced rates for multiple subscriptions.

Prospectus post free on request.

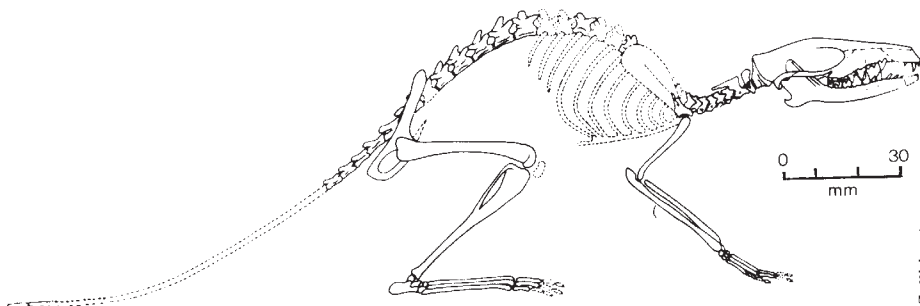
Quarterly List of New Books and new editions added to the Library sent post free to subscribers regularly.

H. K. LEWIS & Co. Ltd.

136 GOWER STREET,
LONDON, WC1E 6BS

Telephone: 01-387 4282
Telegrams: "Publicavt",
London, WC1E 6BS."

Circle No. 11 on Reader Enquiry Card.



Skeletal reconstruction of
Zalambdalestes lechei

Z. Kielan-Jaworowska

provides a complete listing of the taxonomic hierarchy from order to species, giving their authors, and another table shows the known distribution of the genera in space and time. The tables are followed by sections devoted to the anatomy, systematics, phylogeny and ecology of the order. There is also a brief chapter evaluating current opinion on the relationship between the monotremes and the Mesozoic groups.

The taxonomy of Mesozoic mammals used in the book is based on that of Simpson and Romer, and the editors have wisely decided that this was neither the place nor the time to embark on novel ventures in this field. Indeed, they have been extremely cautious, to the extent of refraining from formal use of the terms Prototheria and Theria in view of increasing scepticism about the theory that there was a corresponding fundamental dichotomy in early mammalian evolution. The book can therefore be used without needing to translate one's memory of well-established usage into some new-fangled (and perhaps transient) system of taxonomic/phyletic interpretation, which is a considerable relief.

Three evolutionary topics have been selected for treatment in particular detail.

Crompton and Jenkins discuss the origin of mammals from mammal-like reptiles. The gap between the two groups has been gradually reduced, and little change would be needed in the braincase, jaw-joint and dentition of the small chiniquodont cynodonts known from the Middle Triassic to turn them into the tiny, insectivorous morganucodont mammals of the late Triassic. Crompton and Jenkins believe that, although there are some differences between the structure of the braincase in the therian and non-therian mammals, these are not great, and certainly not so great as to suggest that these two groups originated from the mammal-like reptiles separately.

Bown and Kraus give a clear, well-illustrated account of the origin of the tribosphenic molar, describing how it evolved from the three surfaces of the late Triassic symmetrodonts to the six surfaces of the early eutherians by the gradual proliferation of shearing surfaces. The proliferation was one aspect of the change in tooth shape that exploited the new masticatory process based on transverse shear rather than on simple puncture-crushing.

In the last of these three chapters, Lillegraven discusses reproduction in

Mesozoic mammals. He provides a strong case for believing that it was the evolution of a trophoblast that could suppress maternal immunological rejection of the fetus that provided the eutherians with a decisive advantage over the marsupials. He also suggests that the pair-bonding or social structure, that in eutherians may have facilitated rapid genetic change and evolution, was impossible in marsupials because of the greater likelihood of maternal rejection of subsequent inseminations by the same, or a closely related, male.

At the end of the book Lillegraven, Kraus and Bown summarize the palaeogeographical changes that took place in the Mesozoic world. As they point out, it is in the late Cretaceous that the mammalian fossil record becomes most complete, and therefore the possibility of accurate biogeographical interpretation becomes greatest. But, even here, a good record is known from only two land areas, and only a few fragments are known from three others; in the remaining nine areas the fossil record is silent. Their concluding phrase — "We have much to learn" — may be taken as a suitable keynote, not only for this chapter, but for the book as a whole.

The book has an attractive format, most of the figures are clear and the editors are to be congratulated on an almost fault-free text. Though it would have been improved by the addition of an index, the organization of the book makes it easy to find topics and make cross-references. *Mesozoic Mammals* is an extremely valuable statement and synthesis of this field, both for those whose research lies in or near to it and also for those who have to try to explain it in the course of their teaching. □

Barry Cox is Professor of Zoology at King's College, University of London, UK.

Direct methods and the phase problem

C. J. Gilmore

Theory and Practice of Direct Methods in Crystallography. Edited by M. F. C. Ladd and R. A. Palmer. Pp.421. (Plenum: New York and London, 1980.) \$35, £22.05.

DURING THE past 20 years, three developments have revolutionized X-ray crystallography. Two of them are well known even to the non-specialist. The automatic diffractometer has removed the drudgery of measuring the complicated diffraction pattern that results from the interaction of X-rays and crystals while, at

the same time, increasing the speed and accuracy of the process. Similarly, processing this raw data to produce a crystal structure at an atomic level was also once an even more laborious business that has now been almost completely taken over by computers. As a result of these two advances over 2000 new crystal structures are reported each year; 20 years ago the figure was nearer 200.

One stumbling block still remains. The step between the diffraction experiment and obtaining the crystal structure is not trivial, since the relative phases of the diffracted X-ray beams need to be determined and they cannot be measured experimentally. This is the crystallographic phase problem and it is here that there has been a third major development that is less well known.

It is called 'direct methods' and is a technique by which phase angle

information is obtained directly from the diffraction pattern by mathematical means. The subject started modestly enough 30 years ago as a mathematical curiosity and has grown to become the principal method by which the phase problem is now solved. It has allowed crystallographers to study a whole range of molecules that were previously inaccessible, and it has revolutionized our knowledge of structural chemistry.

It is by no means a static field and there has been a large influx of new ideas in the last decade, but strangely there have been very few books on the subject. Pioneering monographs by Hauptman and Karle (American Crystallographic Association: New York, 1953) and later Woolfson (Oxford University Press: Oxford, 1961) are now outdated or unavailable, and more recent books have either been conference proceedings or have restricted themselves

to specialized aspects of the technique. Consequently, there is a need among crystallographers for a book that covers both theoretical and practical features of direct methods in an integrated and up-to-date way.

This book does not quite achieve this. It contains ten chapters that discuss various aspects of direct methods, each chapter having a separate author. The editorial hand could have been firmer. Apart from the inevitable problems of duplication and overlap, each contributor has been allowed to use his own nomenclature which is consequently different in every section. This will confuse the non-expert and so limit the overall usefulness of the book. The index, too, is rather poor.

There are, however, some excellent individual contributions. Rogers has written a thorough chapter on origin and enantiomorph definition, looking at what can be a difficult subject in a fresh and interesting way. Hauptman, who was one

of the pioneers of direct methods, has contributed a clear and elegant summary of some recent theoretical developments including potentially important work on seminvariant theory. Another pioneer, Sayre, gives an account of phase expansion and refinement methods in his typically concise and illuminating style. There is also an interesting chapter by Argos and Rossmann on the molecular replacement method although its inclusion somewhat extends the customary boundaries of direct methods.

Whether these chapters justify buying the whole volume is of course a personal decision. In any case, the price is not unreasonable for a book of this length. However, the field is still open for a definitive book on direct methods in X-ray crystallography. □

C. J. Gilmore is a Lecturer in Chemistry at the University of Glasgow, UK.

A wealth of virus lore

D.H. Watson

Introduction to Virology. By K. M. Smith and D. A. Ritchie. Pp.212. (Chapman and Hall: London and New York, 1980.) Hardback £12, \$29.95; paperback £5.95, \$14.95.

THE STRENGTH of this book lies in its wide-ranging selection of examples from the world of plant and invertebrate as well as of vertebrate and bacterial viruses. Fungal, protozoal, mycoplasma and spiroplasma viruses are also mentioned. The reader will also benefit from K. M. Smith's wide knowledge of the natural history of plant and insect viruses in particular, as well as from the beautiful electron micrographs (more profuse than is usual in a book of this size) which reach the excellent standard one would expect from this author. Many of these micrographs are of viruses not often represented in illustrations of more comprehensive texts.

The order of presentation is a little unconventional and in some respects illogical; an elementary introduction to the general characteristics of viruses and a survey of the morphology of some representative viruses are followed by chapters on purification, classification and nomenclature, virus diseases of a wide-ranging selection of organisms, spread of viruses and latency, satellite viruses and viroids.

At this stage are interpolated two basic chapters by Professor D.A. Ritchie on virus replication and virus genetics. The chapter on replication in particular is a masterly summary of replication of the various groups of viruses. However, many

teachers of virology would, I think, agree that this core topic should have been brought forward since an understanding of the basic principles of replication is a necessary preliminary to the study of many of the topics discussed in earlier chapters, for example latency.

There follows a rather dated discussion of tumour viruses which only mentions virus isolation and electron microscopy as methods for establishing virus aetiology. Late in the book come tissue and cell culture of viruses and virus assay which are surely basic to much of the earlier chapters.

The book concludes with a chapter on control of virus disease. This is sketchy in the extreme and also somewhat dated. The student reading the paragraphs on interferon will surely contrast the low-key treatment of interferon with what he picks up from the media. The selection of thiosemicarbazone as the only chemotherapeutic agent discussed for animal viruses is also likely to give a somewhat distorted view.

The student requiring an introduction to the subject, either from the standpoint of another discipline or as background reading for an elementary course, would perhaps be better served by one of the other paperback volumes — say Primrose's *Introduction to Modern Virology* (Blackwell Scientific: Oxford) now appearing in a second edition, although Ritchie's chapter on replication would be very usefully read by him. Paradoxically, the book should perhaps be read by more advanced students because of the wealth of lore on viruses often perfunctorily treated in the more standard texts, and also for the wide selection of micrographs. □

D. H. Watson is Professor of General Microbiology and Head of the Department of Microbiology in the University of Leeds, UK.

Mathematical plasma physics

R.J. Bickerton

Plasma Physics for Nuclear Fusion. By K. Miyamoto. Pp.610. (MIT Press: Cambridge, Massachusetts, 1979.) \$50, £31.

PROFESSOR Miyamoto's book is aimed at postgraduate or advanced undergraduate students but it will be equally useful as a reference volume for research physicists in the field. The book was first published in Japanese in 1976 and has now just appeared in English. It appears to date effectively from about 1974 and is consequently dated in the more rapidly moving parts of the subject.

The book opens with arguments in favour of nuclear fusion based on apparently unsubstantiated figures for future energy requirements and for reserves of fossil fuels and uranium. There are then four sections in the book covering fundamentals, magnetohydrodynamics, kinetic description and finally heating diagnostics and confinement. Under fundamentals we have particle orbits, field configurations, Coulomb collisions and the Vlasov equation. The magnetohydrodynamic section includes toroidal equilibria of various kinds, plasma diffusion and a full range of instabilities. Topics considered under kinetic description are waves in cold and hot plasma, Landau damping and microinstabilities due to inhomogeneities in real or velocity space. The final section covers heating by a variety of methods, plasma diagnostic techniques and a survey of confinement experiments.

Professor Miyamoto's view of the world is clearly mathematical rather than physical. The treatment is comprehensive but monochrome with no attempt made to highlight important concepts and no concessions are made or detail sacrificed in the interests of clarity. In many cases the mathematical working is fully displayed so that derivations can be followed step by step. One result is that the book is a heavy read and less than gripping. The main body of the book is very weak and largely silent on the extent to which the theory being presented has been tested by experiment. Although it can be justified by the title it is nevertheless sad that that reader is given no impression of the relevance of the main body of plasma physics to non-fusion fields, in particular to astrophysics.

The chief merit of the book is its completeness and in the detail of the presentation. As such it will be a useful reference book, although somewhat marred by a crop of misprints. □

R.J. Bickerton is Associate Director (Science) at the JET Joint Undertaking, Abingdon, UK.

BOOKS RECEIVED

Mathematics

GROSS J. F. and POPEL, A. (eds) *Mathematics of Microcirculation Phenomena*. Pp. xi + 174. ISBN-0-89004-449-X. (Raven Press, New York, 1980.) \$22.00.

LEITHOLD, L. *College Algebra*. Second edition. Pp. xi + 496. ISBN-0-02-369580-3. (Macmillan Publishing Co., Inc. New York; Collier Macmillan Publishers, London, 1980.) £10.95.

Astronomy

GEHRELS, T. *Asteroids*. Pp. xi + 1181. ISBN-0-8165-0695-7. (The University of Arizona Press, Tucson, Arizona, 1979.) \$19.95.

MOORE, P. *The Pocket Guide to Astronomy*. Pp. 144. ISBN-0-671-25309-3. (Simon & Schuster, New York, 1980.) \$5.95 limp.

Physics

BARDOS, C. and BESSIS, D. (eds) *Bifurcation Phenomena in Mathematical Physics and Related Topics*. Proceedings of the NATO Advanced Study Institute held at Cargèse, Corsica, June, 1979. NATO Advanced Study Institutes Series. Pp. ix + 596. ISBN-90-277-1086-4. (D. Reidel Publishing Company, Dordrecht, Boston, London, 1980.) Dfl. 115 US \$59.50.

CHILD, M. S. (ed.) *Semiclassical Methods in Molecular Scattering and Spectroscopy*. Proceedings of the NATO ASI held in Cambridge, England, September, 1979. NATO Advanced Study Institutes Series. Pp. xi + 332. ISBN-90-277-1082-1. (D. Reidel Publishing Company, Dordrecht, Boston, London, 1980.) Dfl. 75, US\$39.50.

CONSTANTINSCU, F. *Distributions and their Applications in Physics*. International Series in Natural Philosophy Volume 100. Pp. x + 148. ISBN-0-08-018297-6. (Pergamon Press, Oxford and New York, 1980.) £13.50 \$30.00.

DUMMER, G. W. A. (Editor-in-Chief) *Semiconductor and Microprocessor Technology 1979*. Selected papers presented at the 1979 annual SEMINEX Technical Seminar and Exhibition, London. Microelectronics and reliability. Volume 19, No. 5/6. Pp. 407-644. ISBN-0-08-026134-5. (Pergamon Press, Oxford and New York, 1980.) £23.00 flexi.

MARLOW, A. R. *Quantum Theory and Gravitation*. Pp. x + 267. ISBN-0-12-473260-7. (Academic Press, New York and London, 1980.)

PAMPLIN, B. R. (ed.) *Molecular Beam Epitaxy*. Pp. v + 174. ISBN-0-08-025050-5. (Pergamon Press, Oxford and New York, 1980.) US \$35.00 £15.75.

Chemistry

BARD, A. J. and LUND, H. (eds) *Encyclopaedia of the Elements: Organic Section*, volume xiv. Pp. xii + 308. ISBN-0-8247-2514-X. (Marcel Dekker, Inc. New York and Basel, 1980.) Sfr. 240.

BLYTHE, A. R. *Electrical properties of polymers*. Pp. x + 191. ISBN-0-521-21902-7. hardback ISBN-0-521-29825-3 paperback. (Cambridge University Press, Cambridge, 1980.) £4.50 pbk.

CALLEWAERT, D. M. and GENYEA, J. *Basic Chemistry: General, Organic, Biological*. Pp. xxii + 837. ISBN-0-87901-130-0. (Worth Publishers, Inc. New York, 1980.)

EYRING, H., LIN, S. H. and LIN, S. M. *Basic Chemical Kinetics*. A Wiley-Interscience Publication. Pp. vii + 439. ISBN-0-471-05496-8. (John Wiley & Sons, New York and Chichester, UK, 1980.) £20.00.

GOFFER, Z. *Archaeological Chemistry. A Sourcebook on the Applications of Chemistry to Archaeology*. Chemical Analysis: Vol. 55. A Series of Monographs on Analytical Chemistry and its Applications. A Wiley-Interscience Publication. Pp. xvi + 376. ISBN-0-471-05156-X. (John Wiley & Sons, New York and Chichester, UK, 1980.) £15.80.

MURPHY, D. B. and ROUSSEAU, V. *Foundations of College Chemistry*. Third Edition. Pp. xvi + 767. ISBN-0-471-04621-3. (John Wiley & Sons, New York and Chichester, UK, 1980.) £13.00.

SHRINER, R. L., FUSON, R. C. The late, CURTIN, D. Y. and MORRILL, T. C. *The Systematic Identification of Organic Compounds. A Laboratory Manual*. 6th edition. Pp. xviii + 604. ISBN-0-471-78874-0. (John Wiley & Sons, New York and Chichester, UK, 1980.) £13.75.

Technology

BANKS, R. C., MATJEKA, E. R. and MERCER, G. *Introductory Problems in Spectroscopy*. Pp. 326. ISBN-0-8053-0572-6. (Addison-Wesley: 1980.) flexi £9.

GUENOD, M. A. (ed.) *Computer Aided Design of Control Systems*. Proceedings of the IFAC Symposium, Zürich, Switzerland, August, 1979. Pp. 688. ISBN-0-08-024488-2. (Pergamon: 1980.) £125.55.

KIDD, C. V. *Manpower Policies for the Use of Science and Technology in Development*. Pergamon Policy Studies on Socio-Economic Development. Pp. 183. ISBN-0-08-02124-2. (Pergamon: 1980.) £10, \$20.

Earth Sciences

BOOTH, B. and FITCH, F. *La Terre en Colère. Les Cataclysmes Naturels*. Pp. 328. ISBN-2-02-005578-3. (Editions du Seuil, Paris, 1980.) np. flexi.

MACKENZIE, W. S. and GUILFORD, C. *Atlas of rock-forming minerals in thin section*. Pp. v + 98. ISBN-0-582-45591-X. (Longman Group Ltd., Harlow, Essex, 1980.)

CADIOT, B. et al. *Les tremblements de terre en France. Memoire du Bureau de recherches géologiques et minières No. 96-1976*. Pp. ii + 220. (Orleans: Bureau de Recherches Géologiques et Minières, 1979.) F80.00 app. US\$20. flexi.

GOULD, S. J. *Ever Since Darwin: Reflections in Natural History*. Pp. 285. ISBN 0-14-02-222-7. (Penguin: London, 1980.) £1.50.

GUTIERREZ, L. T. and FEY, W. R. *Ecosystem Succession: General Hypothesis and a Test Model of a Grassland*. Pp. 231. ISBN 0-262-07075-8. (MIT; Cambridge, Massachusetts, 1980.) \$25.00.

HARRIS, A. L., HOLLAND, C. H. and LEAKE, B. E. *The Caledonides of the British Isles Reviewed*. Pp. 768. ISBN 0-7073-0257-9. (Scottish Academic Press: Edinburgh, 1980.) £40.00; \$100.00.

JEANS, C. V. and RAWSON, P. J. *Andros Island, Chalk and Oceanic Oozes: Unpublished work of Maurice Black*. Pp. 100. ISBN 0143-6635. (Yorkshire Geological Society: Leeds, 1980.)

KILDOW, J. D. *Deepsea Mining*. Pp. 251. ISBN 0-262-11-075-X. (MIT Press: Cambridge, Massachusetts, and London, 1980.)

KUNKEL, G. *Taxonomic aspects of African economic Botany*. Proceedings of the IX Plenary Meeting of A.E.T.F.A.T. Las Palmas de Gran Canaria, March, 1978. Pp. 250. ISBN-84-500-3340-3. 6kw: The Secretary, Bentham-Moxon Trust, Royal Botanic Gardens, 1980. £11.00.

LONG, C. E. *Discovering The Universe*. Pp. 511 ISBN-0-06044034-1 (Harper and Row: London and New York, 1980.) \$14.95 Flexi.

MORAN, J. M., MORGAN, D. and WIERSMA, J. H. *Introduction to Environmental Science*. Pp. 658. ISBN 0-7167-1020-X (Freeman: Reading, 1980.) £9.20.

MORNER, N. A. (ed.) *Earth Rheology Isostasy and Eustasy*. Pp. 599. ISBN 0-471-27593-X. (John Wiley & Sons: Chichester, 1980.)

POINTINGS, G. and THOMPSON, F. *Hebridean Naturalist: Magazine of the Western Isles Natural History Society*. Pp. 72. ISBN. (Western Isles Natural History Society: Isle of Lewis, 1980.)

OWEN, D. F. *What is Ecology?* 2nd Ed. Pp. 234. ISBN 0-19-219155-1 (Oxford University Press: Oxford, 1980.) £6.50 hardback; £3.50 paperback.

REED, G. M. *Surveys in General Topology*. Pp. 555. ISBN 0-12-584960-5. (Academic: New York and London, 1980.) \$35.00.

RORISON, I. H. and HUNT, R. (eds) *Amenity Grassland. An Ecological Perspective*. Pp. xi + 261. ISBN 0-471-27666-9. (Chichester, New York, Brisbane, Toronto: John Wiley & Sons, 1980.) £19.00.

ROSE, A. W., HAWKES, H. E. and WEBB, J. S. *Geochemistry in Mineral Exploration*. Second ed. Pp. xvii + 657. ISBN-0-12-596250-9 cased ISBN-0-12-596252-5 paperback. (London, New York Toronto, Sydney, San Francisco: Academic Press, 1979.) \$64.50 £28.00 Clothbound \$29.00 £12.40 paperback.

SCHOPF, T. J. M. *Paleoceanography*. Pp. 341. 0-764-65215-0 (Harvard University Press: London and Massachusetts, 1980.) £15.00.

TROMP, S. W. *Biometeorology: The Impact of the Weather and Climate on Humans and Environment (Animals and Plants)*. Pp. xiv + 346. ISBN-0-85501-453-9. Heyden International Topics in Science. (London, Philadelphia, Rhein: Heyden, 1980.) £9.00 \$18.00 DM41.50.

WRATTEN, S. D. and FRY, G. L. A. *Field and Laboratory Exercises in Ecology*. Pp. x + 227. ISBN-0-7131-2735-2. (London: Edward Arnold (Publishers) Ltd., 1980.) £7.50 pbk.

Biological Sciences

ALTMANN, J. *Baboon Mothers and Infants*. Pp. 242. ISBN 0-674-015856-9 (Harvard University Press: Cambridge, Massachusetts and London, England, 1980.) \$17.50.

BRASIER, M. D. *Microfossils*. Pp. 193. ISBN 0-04-562001-6 (George Allen & Unwin: Hemel Hempstead, 1980.) £12.00 Hbk; £6.50 pbk.

CLOUDSLEY-THOMPSON, J. L. *Biological Clocks: Their Functions in Nature*. Pp. 138. ISBN 0-297-77685-1 (Weidenfeld & Nicolson: London, 1980.) £8.95.

DIXON, F. J. and KUNKEL, H. G. (eds) *Advances in Immunology*. Vol. 28. Pp. 532. ISBN 0-12-022428-3 (Academic: London and New York, 1980.) \$37.50.

FOLEY, C. W., LASLEY, J. F. and OSWEILER, G. D. *Abnormalities of Companion Animals: Analysis of Heritability*. Pp. viii + 270. ISBN-0-8138-0940-1. (London: Bailliere Tindall, 1980.) £10.00.

FRANKLIN, E. C. (Editor-in-Chief) *Clinical Immunology Updated: Reviews for Physicians*. Pp. 351. ISBN 0-444-00312-6. (Elsevier/North-Holland: New York, 1980.) \$24.95.

GILULA, N. B. (ed.) *Membrane-Membrane Interactions*. Society of General Physiologists series Volume 34. Pp. xii + 218. ISBN-0-89004-377-9. (New York: Raven Press, 1980.) \$27.00.

GOLDSTEIN, M., CALNE, D. B., LIEBERMAN, A. and THORNER, M. O. (eds) *Ergot Compounds and Brain Function: Neuroendocrine and Neuropsychiatric Aspects*. Advances in Biochemical Psychology Vol. 23. (Raven: New York, 1980.) \$39.00.

GREGORIADIS, G. and ALLISON, A. C. (eds) *Liposomes in Biological Systems*. Pp. 412. ISBN 0-471-27608-1. (John Wiley & Sons: Chichester, 1980.) £20.00.

GRIFFITHS, B., HENNESSEN, W., HORODNICEANU, F. and SPIER, R. *Proceedings of the General Meeting of European Society of Animal Cell Technology*. Vol. 46. Pp. xii + 316. ISBN 3-8055-0764-X. (Karger: Basel and New York, 1980.) Sfr. 70.00; DM 84.00 US \$42.00.

HOCHACHKA, P. W. *Living without Oxygen. Closed and Open Systems in Hypoxia Tolerance*. Pp. xi + 181. ISBN-0-674-53670-3. (Cambridge, Mass, London: Harvard University Press, 1980.) \$17.50.

HORN, A. S., KORF, J. and WESTERINK, B. H. C. (eds) *The Neurobiology of Dopamine*. Pp. xvii + 723. ISBN-0-12-354150-6. (London, New York, San Francisco: Academic Press, 1979.) \$83.00.

HORTON, J. D. (ed.) *Development and Differentiation of Vertebrate Lymphocytes: Developments in Immunology Vol. 8*. Pp. 280. ISBN 0-444-80195 (Elsevier/North-Holland, 1980.)

HUBINONT, P. O. (series editor) *Progress in Reproductive Biology: Seasonal Reproduction in Higher Vertebrates Vol. 5*. R. J. Reiter, San Antonio, Tex. B. K. Follett, Bristol. Pp. xii + 222. ISBN 3-8055-0246-X. (Karger: Basel, New York and Paris, 1980.) Sfr. 155.00; DM 186; US \$93.00.

JEANNEROD, M. and HECKEN, H. *Adaptation et restauration des fonctions nerveuses*. Pp. 392. ISBN 2-85334-178-X. (Simep: Villeurbanne, France, 1980.)

KEENLEYSIDE, M. H. A. *Diversity and Adaptation in Fish Behaviour*. Pp. xiii + 208. ISBN 3-540-09587-X. (Springer: Berlin, Heidelberg and New York, 1979.) DM 69.00; US \$38.00.

KEETON, W. T. *Biological Science: 3rd Edition*. Pp. 1079. ISBN 0-393-95021-2. (W. W. Norton: New York and London, 1980.) \$19.95.

KERR, F. W. L. et al. (Work Session Reports.) *Neurosciences Research Program Bulletin*. Volume Sixteen. Pp. iii + 661. ISBN-0-262-14033-0. (MIT Press: Cambridge, Massachusetts, and London, 1980.) \$30.

LEWIS, W. H. (ed.) *Polyplody: Biological Relevance*. Basic Life Sciences volume 13. Pp. xii + 583. ISBN-0-306-40358-7. (New York and London: Plenum Press, 1980.) \$55.00.

McFADDEN, C. H. *Study Guide for the Third Edition of Keeton's Biological Science*. Pp. 232. ISBN 0-393-95208-X. (W. W. Norton: New York and London, 1980.) \$4.95.

MEISAMI, E. and BRAZIER, M. A. B. (eds) *Neural Growth and Differentiation*. International Brain Research Organization Monograph Series Volume 5. Pp. xvii + 528. ISBN-0-89004-378-7. (New York: Raven Press, 1979.) \$48.00.

MISHELL, B. B. and SHIIGI, S. M. (eds.) *Selected Methods in Cellular Immunology*. Pp. xxix + 486. ISBN-0-7167-1106-0. (San Francisco: W. H. Freeman and Company, 1980.) \$29.95.

MOORE, T. C. *Biochemistry and Physiology of Plant Hormones*. Pp. xii + 274. ISBN-3-540-90401-8. (Berlin, Heidelberg, New York: Springer-Verlag, 1979.) DM 49 approx. US \$27.00.

OGDEN, C. G. and Hedley, R. H. *An Atlas of Freshwater Testate Amoebae*. Pp. 222. ISBN 0-19-858502-0. (Oxford University Press: Oxford, 1980.) £17.50.

PEPE, F. A., SANGER, J. W. and NACHMIAS, V. T. (eds) *Motility in Cell Function*. Proceedings of the First John M. Marshall Symposium in Cell Biology. Pp. xxvi + 479. ISBN-0-12-551750-5. (New York, London, Toronto, Sydney, San Francisco: Academic Press, 1979.) \$28.50.

PEPEU, G., KUCHAR, M. J. and ENNA, S. J. *Receptors for Neurotransmitters and Peptide Hormones: Advances in Biochemical Psychopharmacology*. Vol. 21. Pp. 516. ISBN 0-89004-408-2. (Raven: New York, 1980.) \$48.00.

- PITT, J. I. The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*. vii + 634. ISBN 0-12-557750-8. (Academic: London and New York, 1980.) \$92.00; £40.00.
- PONTING, K. Sheep of the World. Pp. 155. ISBN-0-7137-0941-3. (Poole: Blandford Press, 1980.) £5.95.
- PRASAD, K. N. Regulation of Differentiation in Mammalian Nerve Cells. Pp. xiii + 245. ISBN-0-306-40365-X. (New York and London: Plenum Press, 1980.) \$24.50.
- PROCTOR, J. and PROCTOR, S. Color in Plants and Flowers. Pp. 116. ISBN-0-89696-017-X. (New York: Everest House, 1978.) \$9.95.
- RIDDLE, P. N. Time-Lapse Cinemicroscopy. Biological Techniques Series. ISBN 0-12-58060-X (Academic: London and New York, 1979.)
- ROBINSON, J. P. The Effects of Weapons on Ecosystems. UNEP Studies Series. Pp. ix + 70. ISBN-008-025656-2. (Oxford, New York, Toronto, Sydney, Paris, Frankfurt: Pergamon Press, 1979.) \$10.00 £4.55.
- RONALD, K. and DUGUY, R. (eds) The Mediterranean Monk Seal. UNEP Technical Series Volume 1. Pp. viii + 178. ISBN-0-08-025654-6. (Oxford, New York, Toronto, Sydney, Paris, Frankfurt: Pergamon Press, 1979.) \$20.00 £9.00.
- SCHMALZL, F. and HELLRIGEL, K. P. (eds) Preleukemia. Pp. 194. ISBN 3-540-09798-1. (Springer: Berlin, Heidelberg and New York, 1979.) DM 48.00; US \$26.90 Pbk.
- SCHULSTER, D. and LEVITZKI, A. (eds) Cellular Receptors for Hormones and Neurotransmitters. Pp. 412. ISBN 0-471-27682-0 (John Wiley & Sons: Chichester, 1980.) £25.00.
- SHANKS, R. G. (ed.) Methods in Clinical Pharmacology-Cardiovascular System. Pp. 106. ISBN-0333-28282-5. (Basingstoke: Macmillan Publishers Ltd. Scientific & Medical Division, 1980.) Hardback £10.00.
- SPAWLS, S. Sun, Sand and Snakes. Pp. 254. ISBN-0-00-262760-4. (London: Collins and Harvil Press, 1980.) £6.95.
- VOGEL, de E. F. Seedlings of Dicotyledons. Pp. 465. ISBN 90-220-0696-4 (Centre for Agricultural Publishing and Documentation: The Netherlands, 1980.) Dfl. 150.00.
- WELLS, N. Medicines: 50 years of Progress 1930-1980. Pp. 60. (Office of Health Economics, Free of Charge at the Economics of Health Office, 1980.)
- WOOLEY, D. E. and EVANSON, J. M. (eds) Collagenase in Normal and Pathological Connective Tissues. Pp. 292. ISBN 0-471-27668-5 (Wiley Interscience: Chichester, 1980.) £16.00.
- WUTTKE, W., WEINDLE, A., VOIGT, K. H. AND DRIES, R. R. Brain and Pituitary Peptides: Ferring Symposium on Brain and Pituitary Peptides Munich 1979. Pp. vii + 240. ISBN 3-8055-0165-X. (S. Karger: Basel, New York and München, 1980.)

Applied Biological Sciences

- ALFIN-SLATER, R. B. and KRICHEVSKY, D. (eds) Human Nutrition: Nutrition and the Adult Macronutrients 3A. Pp. 290. ISBN 0-306-40287-4 (Plenum: New York and London, 1980.)
- ARIENS, E. J. (ed.) Drug Design. Vol IX. Pp. 355. ISBN 0-12-060309-8. (Academic: New York and London, 1980.) \$39.50.
- BACH-y-RITA, P. (ed.) Recovery of function: Theoretical considerations for brain injury rehabilitation. Pp. 278. ISBN 3-456-890836-4. (Hans Hurer Publisher: Berne, Stuttgart and Vienna, 1980.) Fr. 34.00; DM 37.00 Pbk.
- BLOOR, C. M. and LIEBOW, A. A. The Pulmonary and Bronchial Circulations in Congenital Heart Disease. Topics in Cardiovascular Disease. Pp. 279. ISBN 0-306-40383-8. (Plenum: New York, 1980.) \$32.50.
- COHEN, E. N. Anaesthetic Exposure in the Workplace. Pp. 197. ISBN 0-85200-348-X. (MTP: Lancaster, UK, 1980.) £11.75.
- COMMONWEALTH BUREAU OF NUTRITION. Anorexia nervosa. Annotated Bibliography (1973-79) Pp. i + 179 ISBN 0-141-5441 (CNS, Bucksburn, Aberdeen, 1980.) £7.50.
- COMMONWEALTH BUREAU OF NUTRITION. Aquaculture. Annotated Bibliography (1973-79) Pp. i + 179 ISBN 0-141-5441 (CNS, Bucksburn, Aberdeen, 1980.) £7.50.
- DALTON, C. D. An Introduction to Practical Animal Breeding. Pp. 162. ISBN 0-246-11351-0. (Granda Publishing: St. Albans, 1980.) £4.95 Pbk.
- EDDY, D. M. Screening for Cancer: Theory, Analysis, and Design. Pp. 308. ISBN 0-13-796789-6. (Prentice-Hall: 1980.) £19.45.
- GETZ, M. E. Getz, Paper & Thin Layer Chromatographic Analysis of Environmental Toxicants. Pp. 164. ISBN 0-85501-451-2. (Heyden: London, Philadelphia and Rheine, 1980.) £7.30; \$16.50; DM 34.50.
- GNANAM, A., KRISHNASWAMY, S. and KAHN, J. S. Biological Applications of Solar Energy: Proceedings of the International Symposium on. Pp. 214. (Macmillan India: Madras, 1980.) Rs. 75.00.
- KLEIN, G. (ed.) Viral Oncology. Pp. 860. ISBN 0-89004-390-6. (Raven: New York, 1980.) \$85.00.
- MANCINI, M. et al. Medical Complications of Obesity; Proceedings of the Serna Symposia. Vol. 26. Pp. 406. ISBN 0-12467150-0. (Academic: New York and London, 1980.) \$43.50; £18.80.
- NEEDHAM, J. R. Handbook of Microbiological Investigations for Laboratory Animal Health. Pp. xviii + 174. ISBN 0-12-515950-1. (Academic: New York and London, 1980.) £9.80 \$21.00.
- OLIVER, J. S. Forensic Toxicology: Proceedings of the European Meeting of the International Association of Forensic Toxicologists. Pp. 320. ISBN 0-7099-0362-6 (Croom Helm Ltd: London, 1980.) £14.50.
- OPENSHAW, K. Cost and Financial Accounting in Forestry: Practical Manual Pp. 188. ISBN 0-08-021455-X. (Pergamon: Oxford and New York, 1980.) \$13.75; £6.25 Pbk \$27.50; £12.50 Board.
- PLAATS, G. F. van der. Medical X-Ray Techniques in Diagnostic Radiology. Fourth Edition. Pp. 463. ISBN 0-333-26415-0 (Macmillan: London, 1980.) £35.00.
- READ, S. E. and ZABRISKIE, J. B. Streptococcal Diseases and the Immune Response. Pp. 830. ISBN 0-12-583880-8. (Academic: New York and London, 1980.) \$45.00.
- SHALHEVET, J. et al. (ed.) Irrigation of Field and Orchard Crops under semi-arid conditions. Revised edition. Pp. 110 ISBN 0-08-025511-6. (International Irrigation Information Center: Pergamon Press: Oxford and New York, 1980.) \$9.00; £4.50.
- SOPPER, W. E. and KERR, S. N. (eds) Utilization of Municipal Sewage Effluent and Sludge on Forest and Disturbed Land. Pp. 537. ISBN 271-00205-0. (Pennsylvania State University Press: 1980.) £12.
- TURUSOV, V. S. (Editor-in-Chief) Pathology of Tumours in Laboratory Animals. Vol II. Tumours in the Mouse. Pp. 661. ISBN 92-832-1123-5. (World Health Organisation: International Agency for Research on Cancer, 1980.) Sw. Fr 100.00 US \$60.00.
- WAYNFORTH, H. B. Experiment and Surgical Technique in the Rat. Pp. 265. ISBN 0-12-738850-8. (Academic: London, 1980.) £11.80; £27.50.
- WERTELECKI, W. and PLATO, C. C. (eds) Dermatoglyphics — Fifty Years Later. Pp. 800. ISBN 0-8451-1031-4. (Alan R. Liss: New York, 1980.) \$76.00.

Psychology

- BUTTERWORTH, B. (ed.) Language Production. Vol. 1. Speech and Talk. Pp. x + 478. (Academic: London and New York, 1980.) \$64.50; £28.00.

General

- ALLEN, M. Falconry in Arabia. Pp. 143. ISBN-0-85613-013-3. (London: Orbis Publishing, 1980.) £15.00.
- BOOTH, D., RODIN, J. and MOGENSON, G. Appetite: The Journal for Research on Intake, its Control and its Consequences. Vol. 1. No. 1. March 1980. Pp. 102. ISBN 0195-6663 (Academic: London and New York, 1980.) £27.00 Pbk.
- CAFFERTY, B. and HOOPER, D. A complete Defence to 1P-K4. A Study of Petroff's Defence, Second edition. Pergamon Chess Series. Pp. xiv + 149. ISBN-0-08-024089-5 hardback ISBN-0-08-024088-7 flexi. (Oxford, New York, Toronto, Sydney, Paris, Frankfurt: Pergamon Press, 1979.) \$14.00 £6.50 hardback \$8.00 £3.50 flexi.
- COHEN, M. L., RONEN, N. and STEPAN, J. Law and Science: Selected Bibliography. Pp. 155. ISBN 0-262-03073-X. (MIT: Cambridge, Massachusetts, 1980.) \$15.00.
- COLINVAUX, P. Why Big Fierce Animals Are Rare. How the Natural World Works. Pp. x + 224. ISBN-0-04-574015-1. (London, Boston, Sydney: George Allen & Unwin Ltd., 1980.) £7.95.
- DAVIDGE, R. W. Mechanical behaviour of ceramics. Pp. viii + 165. ISBN-0-521-21915-9 hardback ISBN-0-521-29309-X pbk. (Cambridge, London, New York, New Rochelle, Melbourne, Sydney: Cambridge University Press, 1980.) £3.95 pbk.
- deRIVERO, O. B. New Economic Order and International Development Law. Pp. xvi + 141. ISBN-0-08-024706-7. (Oxford, New York, Toronto, Sydney, Paris, Frankfurt: Pergamon Press, 1980.)
- DUNN, E. K. and BLACK, D. (eds) The Twelve Months of the Year. Pp. 120. ISBN-0-7153-7918-6 hardback ISBN-0-7153-7919-4 paperback. (Newton Abbot: David & Charles, 1980.) £3.95 paperback £5.95, hardback.
- PRENEY, J. R. and NICOLSON, A. J. (eds) Sulfur in Australia. Pp. 268. ISBN-085847-059-4. (Australian Academy of Science: Canberra, 1980.) £26.50.
- GOSSELIN, C. and WILSON, G. Sexual Variations: Fetishism, Transvestism and Sado-masochism. Pp. 169. ISBN-0-571-11538-1. (Faber & Faber: London, 1980.) £7.50.
- GRANT, J. (ed.) The Book of Time. Pp. 320. ISBN-0-7153-7764-7. (Newton Abbot: David & Charles, 1980.) £10.50.
- GRIFFITHS, F. and POLANYI, J. C. (eds) A Pugwash Symposium. Pp. xiii + 193. ISBN-0-8020-2356-8 hardback. ISBN-0-8020-6389-6 hardback.
- GRIFFITHS, F. and POLANYI, J. C. (eds) The Dangers of Nuclear War. A Pugwash Symposium. Pp. xiii + 193. ISBN-0-8020-2356-7 hardback. ISBN-0-8020-6389-6 paperback. (Toronto, Buffalo, London: University of Toronto Press, 1980.) \$15.00 hardback \$5.95 paperback.
- HAKEN, H. (ed.) Pattern Formation by Dynamic Systems and Pattern Recognition. Proceedings of the International Symposium on Synergetics at Schlob Elmau, Bavaria, Springer Series in Synergetics Volume 5. Pp. viii + 305. ISBN 3-540-09770-8. (Berlin, Heidelberg, New York: Springer-Verlag, 1979.) DM68 approx. US \$37.40.
- HAYNES, R. D. H. G. Wells: Discoverer of the Future. The Influence of Science on his Thought. Pp. xi + 283. ISBN-0-333-27186-6. (London and Basingstoke: The Macmillan Press, Ltd., 1980.) £12.00.
- HEIM, A. Barking up the right Tree or Intelligence and Personality in Dogs. Pp. 208. ISBN-0-1214-9. (London: Pelham Books, 1980.) £6.50.
- KRASNEGOR, N. A. (ed.) Behavioural Analysis and Treatment of Substance Abuse. Pp. 256. Stock No. 017-024-00939-3. (Department of Health, Education, and Welfare: National Institute on Drug Abuse Research 25 Monograph Series, Washington, 1980.) £4.50.
- KRASNEGOR, N. A. (ed.) The Behavioural Aspects of Smoking. NIDA Research Monograph Series 26. Pp. 192. Stock No. 017-024-009474. (Department of Health, Education and Welfare: Government Printing Office, Washington, 1980.) \$12.00.
- LUEPKE, N.-P. (ed.) Monitoring Environmental Materials and Specimen Banking. Proceedings of the International Workshop, Berlin (West) October, 1978. Pp. xiii + 391. ISBN-90-247-2303-5. (The Hague: Martinus Nijhoff, 1980.) Guilders 150.
- MANUEL, F. E. A Portrait of Isaac Newton. Pp. 478. ISBN-0-584-95357-7. (Frederick Muller: London, 1980.) £11.75.
- NARNHAM, P. Fantastic Invasion: Dispatches from Contemporary Africa. Pp. 271. ISBN-01-636-5764. (Jonathan Cape: London, 1980.) £6.50.
- NUKI, G. (ed.) The Aetio-pathogenesis of Osteoarthritis. Pp. x + 212. ISBN-0-272-79434-1. (Tunbridge Wells: Pitman Medical Limited, 1980.) £15.00.
- PAARLBERG, D. Farm and Food Policy: Issues of the 80s. Pp. 338. ISBN-0-8032-3656-5. (University of Nebraska Press: Lincoln, Nebraska, 1980.) £9.90.
- SANDBANK, C. P. (ed.) Optical Fibre: Communication Systems. Pp. 347. ISBN-0-471-27667-7. (John Wiley & Sons: Chichester and New York, 1980.) £17.50.
- SCHUTTE, H. R. Wirkungsmechanismen von Herbiziden und synthetischen Wachstumsregulatoren. Pp. 381. ISBN-533-286-9. (VEB Gustav Fischer Verlag, Jena e. Germany, 1980.)
- SCHWARZBACH, M. Alfred Wegener und die Drift der Kontinente. Pp. 160. ISBN-3-8047-0582-0. (Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 1980.) DM29.
- SHAW, T. L. (ed.) An Environmental Appraisal of Tidal Power Stations: with particular reference to the Severn barrage. Pp. 220. ISBN-0-273-08463-1. (Pitman: London, 1980.) £9.75.
- SIPRI: Stockholm International Peace Research Institute. Pp. 201. ISBN-0-85066-199-4. (Taylor & Francis: London, 1980.) £6.50; US \$19.50 pbk.
- STEVENSON, J. P. Trout Farming Manual. Pp. 183. ISBN-0-85238-102-6. (Fishing News Books: Farnham, Surrey, 1980.) £9.75 + 95p p&p.
- STOCKHOLM INTERNATIONAL PEACE RESEARCH INSTITUTE. Nuclear Energy and Nuclear Weapon Proliferation. Pp. xxv + 462. ISBN-0-85066-184-6. (London: Taylor & Francis Ltd., 1979.) £14.00.
- SZERI, A. Z. (ed.) Tribology: Friction, Lubrication, and Wear. Pp. 584. ISBN-007-062663-4. (Hemisphere Publishing Corp: New York, 1980.) \$32.00.
- TURNER, E. M. (ed.) Kapchigai Defile: The Journal of Paul Nazaroff. Pp. 127. ISBN-0-584-10463-4. (Frederick Muller: London, 1980.) £7.95.
- UNITED NATIONS ECONOMIC COMMISSION FOR EUROPE. The Gas Situation in the ECE Region Around the Year 1990. Proceedings of an International Symposium of the Committee on Gas of the Economic Commission for Europe, France, 1978. Pp. xviii + 373. ISBN-0-08-024465-3. (Pergamon: Oxford and New York, 1979.) \$60.00, £27.00.
- UNITED NATIONS ENVIRONMENT PROGRAMME. Directory of Institutions and Individuals Active in Environmentally-Sound and Appropriate Technologies. UNEP Reference Series. Pp. xviii + 152. ISBN-0-08-025658-9. \$20.00, £9.00.
- FREIMAN, G. It Seems I Am a Jew. A Samizdat Essay. Pp. 96. ISBN-0-8093-0962-9. (Southern Illinois University Press: Effner and Simons: 1980.) Np.
- KATSENELINBOIGEN, A. Soviet Economic Thought and Political Power in the USSR. Pergamon Policy Studies on the Soviet Union and Eastern Europe. Pp. 213. ISBN-0-08-022467-9. (Pergamon: 1980.) £12.50.
- SLAPPEY, S. G. (ed.) The Future of Business—Annual Review 1980/81. Pergamon Policy Studies on U.S. and International Business. Pp. 113. Hbk ISBN 0-08-025585-X; Flexi ISBN 0-08-025584-1. (Pergamon: 1980.) Hbk £7.50; flexi £2.70.
- STOCKHOLM INTERNATIONAL PEACE RESEARCH INSTITUTE. World Armaments and Disarmament. SIPRI Yearbook 1980. Pp. 514. ISBN 0-85066-201-X. (Taylor & Francis, 1980.) £19.
- TASCA, D. (ed.) U.S.—Japanese Economic Relations; Cooperation, Competition, and Confrontation. Pergamon Policy Studies on U.S. and International Business. Pp. 135. ISBN 0-08-025129-3. (Pergamon: 1980.) £8.25.